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Purification of *Pseudomonas aeruginosa* Exoenzyme S

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***Pseudomonas aeruginosa* produces two distinct ADP-ribosyl transferases, exotoxin A and exoenzyme S, which differ in a number of properties including substrate specificity. Exoenzyme S was purified from culture supernatants of *P. aeruginosa* DG1. The procedure for purification consists of four major steps: ammonium sulfate precipitation, anion-exchange chromatography on DEAE-Sephacel, acetone precipitation in the presence of 1 M NaCl, and G-100 Superfine gel filtration chromatography. Exoenzyme S was monitored during purification by an assay for ADP-ribosyl transferase activity, mouse toxicity, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified material exhibited ADP-ribosyl transferase activity, reacted with monoclonal antibodies to exoenzyme S, and was toxic to mice and a variety of tissue culture cell lines.**

Pseudomonas aeruginosa elaborates several potentially toxic extracellular products which may contribute to its pathogenicity (26). Included among these are two distinct ADP-ribosyl transferases, exotoxin A (9) and exoenzyme S (11). Liu (15) isolated a heat-labile toxin, designated exotoxin A, from filtrates of *P. aeruginosa* cultures and showed that injection of this into mice produces symptoms similar to those observed in animals infected with live organisms. Iglewski and Kabat (9) demonstrated that exotoxin A is similar in a number of ways to diphtheria toxin. It inhibits protein synthesis by ADP-ribosylation of elongation factor 2 (EF-2) and is synthesized predominantly during the late log phase, and its production is dependent on the concentration of iron in the medium.

A number of *P. aeruginosa* strains produce an enzyme different from exotoxin A which ADP-ribosylates eucaryotic proteins (11). This enzyme, termed exoenzyme S, is distinct from exotoxin A by a lack of neutralization with exotoxin A antisera and by the observation that exoenzyme S does not ADP-ribosylate EF-2, but rather modifies one or more different proteins present in eucaryotic cell extracts (11). Little more is known about the enzymology of exoenzyme S. Although there was an initial indication that one target cellular protein was EF-1 (11), this has not been confirmed with a purified preparation.

Studies of the role of exoenzyme S in the pathogenesis of disease due to *P. aeruginosa* have been hampered by the lack of a purified preparation of the enzyme. Previous attempts to purify exoenzyme S have employed detergents at one or all purification steps, which has in some manner rendered the enzyme nontoxic for experimental animals (23). In the present work, we describe a purification procedure for exoenzyme S which avoids the use of detergents and yields an end product which is toxic to experimental animals and tissue culture cells as well.

MATERIALS AND METHODS

Bacteriological media and growth conditions. Cell-free culture supernatant from *P. aeruginosa* DG1 was used as the source for exoenzyme S. Organisms were grown for 36 h at 32°C in S medium (27). S medium contained the following components (per liter of distilled water): NH_4Cl, 1 g; Na_2HPO_4, 3 g; NaCl, 5 g; $MgSO_4$, 0.1 g; EDTA, 1.7 g; sodium succinate, 27 g. After sterilization by autoclaving, the medium was allowed to cool, and monosodium glutamate and glycerol were added (filter sterilized, 100 mM and 1% final concentrations, respectively). Ten 2-liter baffled flasks containing 500 ml of S medium were each inoculated with 100 μl of cells obtained from a starter culture of *P. aeruginosa* DG1 (18 h, 32°C, A_{550} of 2.5). During the incubation period, cultures were shaken at 200 rpm for maximum aeration. At the end of the incubation period (A_{550} of 1.8), cultures were centrifuged at 10,000 × *g* for 20 min at 4°C to remove cells.

Exoenzyme S purification. To 5 liters of culture supernatant, solid ammonium sulfate was added slowly to 60% saturation. After overnight incubation at 4°C, the material was centrifuged at 15,000 × *g* for 30 min at 4°C to obtain the precipitated protein. The precipitate was dissolved in 100 ml of 0.05 M Tris hydrochloride buffer (pH 8.0) containing 0.01 M NaCl (Tris buffer) and dialyzed overnight at 4°C against 6 liters of this same buffer. The dialyzed material (150 ml, 0.94 mg of protein per ml) was applied to a column (2.6 by 30 cm) of DEAE-Sephacel (Pharmacia, Uppsala, Sweden) equilibrated in Tris buffer. The column was eluted with a linear gradient of 0.01 to 1.01 M NaCl in Tris buffer (total volume, 500 ml). The flow rate was controlled by a peristaltic pump at 40 ml/h, and 8-ml fractions were collected. Protein-containing fractions were detected by measuring the A_{280}. Those fractions eluting at 0.4 to 0.5 M NaCl were pooled; NaCl was added to the pooled material to an approximate final concentration of 1 M, and this material was cooled to 0°C in an ice-salt bath. Acetone (approximately 30 ml) previously cooled to −20°C was added slowly with continuous monitoring of the temperature, which was not allowed to rise above 2°C. When the acetone concentration had reached 33%, the temperature was allowed to fall to 0°C and equilibrated for 15 min. Acetone-precipitable material was collected by centrifugation (20 min, 5,000 × *g*, 0°C). The precipitate was redissolved in 4 ml of Tris buffer and dialyzed overnight at 4°C against 6 liters of this same buffer. The dialyzed material (5.5 ml, 0.4 mg of protein per ml) was applied to a G-100 gel filtration column (2.6 by 77.5 cm; Pharmacia) equilibrated in Tris buffer. The flow rate was

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gravity controlled at 15 ml/h, and 8-ml fractions were collected. Protein-containing fractions were detected by measuring the A_{280}. In separate experiments, EDTA (0.05 M) or phenylmethylsulfonyl fluoride (0.001 M) or both were incorporated into all buffers used in the purification procedure.

Enzyme activity. Crude extracts containing aminoacyl transferase factors were prepared from wheat germ as described by Chung and Collier (6). ADP-ribosyl transferase activity was measured by the incorporation of radioactivity from [adenine-^{14}C]NAD$^+$ trichloroacetic acid-precipitable material in the presence of crude wheat germ extracts as previously described (10). Unless otherwise noted, the reaction was performed at 25°C for 30 min in 0.1 ml of 50 mM Tris hydrochloride (pH 7.0)–1 mM EDTA–50 mM dithiothreitol–0.12 mM [adenine-^{14}C]NAD$^+$ (10.6 mCi/mmol; Amersham Corp., Arlington Heights, Ill.), wheat germ extract containing 150 to 160 μg of proteins, and various amounts of exoenzyme S. The reaction was stopped by the addition of 0.1 ml of 10% trichloroacetic acid, and the precipitates were collected, washed, and counted as previously described (10). One unit of enzyme activity was defined as that amount of enzyme which transferred 4.0 nmol of [adenine-^{14}C]ADP-ribose per min from [adenine-^{14}C]NAD$^+$ into trichloroacetic acid-precipitable material in the presence of crude wheat germ extracts. In additional experiments, purified oubain-sensitive ATPase obtained from Sigma Chemical Co. (St. Louis, Mo.) was substituted for wheat germ extract as the substrate (25).

Protein determination. Protein concentrations were determined by the method of Bradford (4) with a commercially prepared reagent (Bio-Rad Laboratories, Richmond, Calif.).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (14). The gels were cast in 1.5-mm slabs in a 10-cm electrophoresis apparatus (Aquebogue Machine and Repair Shop, Aquebogue, N.Y.). Samples were dissolved in a solution containing 0.5 M Tris hydrochloride (pH 6.8), 1% 2-mercaptoethanol, 2% SDS, and 10% (vol/vol) glycerol and immediately heated at 100°C for 2 min. Electrophoresis was carried out at a constant current of 30 mA per slab for 4 h. After electrophoresis, the gels were fixed and silver stained (20), or in some cases, proteins were transferred from gels to nitrocellulose paper as described below.

Preparation of specific rabbit antiserum to exotoxin A. Exotoxin A was purified as previously described (10). A 1-ml mixture of equal parts of Freund complete adjuvant and exotoxin A protein (300 to 500 μg/ml) was injected three times at 2-week intervals into female New Zealand White rabbits (2 to 2.5 kg). Ten days after the last injection, the rabbits were exsanguinated by cardiac puncture. The serum was separated by centrifugation (15,000 × *g*, 10 min) and stored in aliquots at −70°C. The gamma globulin fraction was obtained from the antiserum by batch binding to DEAE-Sephacel as described previously (3). The material obtained was lyophilized and suspended in phosphate-buffered saline (PBS) to the original volume of antiserum.

Cell culture medium and materials. Dulbecco modified eagle medium with sodium pyruvate (Flow Laboratories, Mississauga, Ontario, Canada) was supplemented with glutamine, penicillin, streptomycin (DMEM), and 10% fetal calf serum (FCS) (Myoclone; GIBCO, Burlington, Ontario). Hypoxanthine-aminopterin-thymidine and hypoxanthine-thymidine (HAT and HT, respectively) media were obtained from Flow Laboratories.

Chinese hamster ovary (CHO) cells. HeLa cells, and mouse L cells were obtained from the American Type Culture Collection (Rockville, Md.). Vero cells were kindly provided by C. H. Pai (University of Calgary). Cell lines were grown in DMEM–10% FCS, and confluent monolayers were removed by treatment with trypsin-EDTA (GIBCO) for 10 min at 37°C. Cells were washed with fresh DMEM–10% FCS and resuspended to approximately 10^5 cells per ml in fresh DMEM–10% FCS.

The myeloma cell line NS-1 (a nonsecreting clone of P3 × 63 Ag 8 [12]) was kindly provided by J. W. Costerton (University of Calgary). These cells were maintained in DMEM–10% FCS. When the cell density reached approximately 10^7 cells per ml, cells were diluted to 1×10^6 to 2×10^6 cells per ml in fresh DMEM–10% FCS and transferred. All cell cultures were maintained at 37°C under 7% CO_2.

Monoclonal antibody production. A purified preparation of exoenzyme S (G-100 eluate material) was used as the immunogen for monoclonal antibody production. Before use as an immunogen, the material (2.0 ml, 200 μg/ml) was dialyzed against 0.1 M Tris buffer (pH 8.0) containing 1% Formalin and 0.2 M L-lysine (Calbiochem-Behring, La Jolla, Calif.) at 37°C for 72 h. The toxoid preparation was then dialyzed against 0.2 M L-lysine–saline solution (0.85% NaCl, pH 6.3) at 4°C for 48 h.

Hybrid cell lines were prepared by published methods (13). BALB/c mice were injected four times intraperitoneally with 50 μg of Formalin-lysine-treated exoenzyme S on days 0, 7, 14, and 21. Three days after the final injections, spleens were removed, and the spleen cells were collected. Spleen and NS-1 cells (10:1) were fused with 50% (wt/wt) polyethylene glycol (molecular weight, 1,500). Fused cells were transferred into microtiter dishes at 6×10^4 input spleen cells per well and maintained for 14 days in HAT medium. The tissue culture supernatant fluids from each microtiter well were then tested by enzyme-linked immunosorbent assay (ELISA) for the presence of antibody to exoenzyme S. Hybridoma cell lines producing monoclonal antibody to exotoxin S were transferred to HT medium and cloned by limiting dilution, and 2×10^6 cells were injected into pristane-primed mice for ascites tumor induction. The hybridoma cell lines were extensively subcloned (six times) to ensure that multiple antibodies were not generated.

Purification of monoclonal antibodies from ascitic fluids was accomplished by hydroxylapatite column chromatography as previously described (22). Ascites fluids were centrifuged at 20,000 × *g* for 30 min at 4°C before chromatographic separation on hydroxylapatite columns. Ascitic fluid (35 ml) was applied to a column (2.5 by 15 cm) of hydroxylapatite (bed volume of approximately 75 ml; HTP grade; Bio-Rad Laboratories) hydrated in column buffer (0.01 M sodium phosphate–0.02% sodium azide, pH 6.8). The bound proteins were then eluted with a 500-ml 0.01 to 0.3 M linear phosphate gradient (pH 6.8) at room temperature. The flow rate was controlled with a peristaltic pump at 60 ml/h, and 4-ml fractions were collected. Antibody activity in column fractions was assayed with partially purified exoenzyme S by ELISA and by Western blot analysis as described below.

Electrophoretic blotting procedure. The reactivity of antibody to exoenzyme S was determined by the electroblotting technique. Protein was electrophoresed as described above and transferred to nitrocellulose as described by Towbin et al. (24) by using a Transblot apparatus (Hoeffer) for 30 min at 1 A. The nitrocellulose was incubated at 37°C for 1 h in 3% bovine serum albumin (BSA) in 10 mM Tris hydrochloride (pH 7.4)–0.9% NaCl (Tris-saline) to block nonspecific binding of antiserum. The nitrocellulose paper was incubated at 4°C with 100 μl of antiserum at a dilution of 1:1,000. The

blots were then incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin and immunoglobulin M (IgM) (Miles Laboratories, Inc., Elkhart, Ind.) at a 1:2,000 dilution in Tris-saline containing 3% BSA for 2 h at 37°C. The blots were then immersed in a solution of 25 μg of *O*-dianisidine per ml, 0.01% H_2O_2, and 10 mM Tris (pH 7.4) as described by Avrameas and Guilbert (2). Positive reactions were noted by the presence of a strong brown color in the area of activity.

ELISA procedure. An ELISA was used to screen for monoclonal antibodies to exoenzyme S. Purified exoenzyme S (10 μg/ml in sodium carbonate, pH 8.8) was dispensed in 200-μl volumes into 96-well microtiter dishes which were incubated overnight at 4°C. The plates were washed three times, incubated for 2 h at 37°C with 200 μl of proteins from 1:2 dilutions of rabbit antitoxin A (specificity control), hybridoma culture medium (medium control), or supernatant fluids containing anti-exoenzyme S antibody, and washed again three times. Peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit IgA and IgM (Miles Laboratories) was diluted to 1:10,000, and 200-μl portions were added to the appropriate wells. The plates were incubated for 2 h at 37°C and again washed. All washes and antibody dilutions were made in PBS containing Tween 20 (Sigma) at a final concentration of 0.05%. Color development was achieved as previously described (5). Additional control wells were treated identically, except that PBS replaced the serum (serum control), the conjugated anti-IgG and anti-IgM (conjugate control), or the substrate (substrate control). Endpoints were wells with an absorbance reading twice that of a serum control. All assay results were examined at 450 nm in a Microelisa Auto Reader (Dynatech Laboratories, Inc., Alexandria, Va.).

Cytotoxicity. Tissue culture monolayers were harvested by rinsing with 5 ml of trypsin-EDTA (GIBCO), incubation for 10 min with 5 ml of trypsin-EDTA at 37°C in 7% CO_2, mixing with an equal volume of cold medium, and centrifugation (5,000 × *g* for 10 min). The cells were resuspended in fresh medium to a density of 10^5 cells per ml, and 200 μl was dispensed into each well of a microtiter plate and incubated overnight at 37°C in 7% CO_2. Various concentrations of the material to be tested were filter sterilized, diluted in DMEM, and added to the cells. After a timed incubation (48 h) at 37°C, cytotoxicity for the cells was determined by measuring exclusion of trypan blue. A 50-μl portion of 0.05% trypan blue in sterile saline was added to each well, and the cultures were incubated at 37°C for an additional 15 min. The supernatants from each well were aspirated and transferred to clean wells in a separate microtiter plate in which the A_{550} of individual wells was determined with a microplate reader. One 50% tissue culture dose was defined as the amount of toxic activity required to increase trypan blue uptake by 50%.

Mouse lethality. Various concentrations of the material to be tested were filter sterilized and diluted in PBS. Samples (1 ml) were injected peritoneally into each of 10 male Swiss Webster mice weighing between 18 and 20 g. The mean lethal dose (LD_{50}) was calculated by the formula of Reed and Muench (21).

Antibody neutralization assays. Monoclonal antibodies to exoenzyme S were tested for their ability to inactivate enzyme activity by assaying ADP-ribosyl transferase activity after incubation with 0.2 mg of purified exoenzyme S. This concentration of exoenzyme S gave approximately 50% maximum activity in the ADP-ribosyl transferase assay. Normal mouse serum and rabbit anti-toxin A antiserum were used as controls. Monoclonal antibody and control serum preparations were adjusted to 100 μg of IgG or IgM per ml and serially diluted in saline containing 0.1 mg of bovine serum albumin. After preincubation with exoenzyme S for 15 min at 37°C, the mixtures were assayed for ADP-ribosyl transferase activity as described above. The highest dilution of each monoclonal antibody preparation which gave less than 50% of control (normal mouse serum) values in the ADP-ribosyl transferase assay was taken as the endpoint.

Monoclonal antibodies to exoenzyme S were tested for their ability to neutralize cytotoxicity by assaying trypan blue uptake by cells exposed to 5 50% tissue culture doses of purified exoenzyme S preincubated with monoclonal antibody preparations for 1 h at 37°C. Normal mouse serum and rabbit anti-exotoxin A antiserum were used as controls. Monoclonal antibody and control serum preparations were adjusted to 100 μg of IgG or IgM per ml and serially diluted in DMEM-10% FCS. The highest dilution of each monoclonal antibody preparation which prevented less than 10% of control (normal mouse serum) trypan blue uptake was taken as the endpoint.

Monoclonal antibody affinity purification of exoenzyme S. A monoclonal antibody affinity column was used to purify exoenzyme S by the method of Hissey et al. (8). Reactigel (10 ml; Pierce Chemical Co., Rockford, Ill.) was washed on a sintered glass filter with 10 volumes of acetone-water (7:3), 10 volumes of acetone-water (3:7), and 10 volumes of 0.1 M borate–0.9% saline, pH 8.5. Dry gel cake (5 ml) was then added to 20 ml of 0.1 M borate–0.9% saline (pH 8.5) containing approximately 2 mg of purified monoclonal antibody to exoenzyme S. Coupling was allowed to proceed for 48 h at 4°C with mixing. Coupled gel was then washed in 2 M Tris hydrochloride (pH 8.0) to block any remaining active sites and stored in PBS at 4°C.

A 5-ml sample of acetone-precipitated, dialyzed exoenzyme S (0.4 mg/ml) was diluted 1:1 in PBS and cycled through a 5-ml affinity column for 24 h. The column was then washed in PBS, and the bound exoenzyme S was eluted in 0.2 M glycine, pH 2.5. Fractions of 1 ml were collected into 100 μl of 2 M Tris, pH 7.0. All procedures were performed at 4°C. Protein-containing fractions were detected by measuring the A_{280}.

Amino acid composition. Partial amino acid analyses of affinity-purified exoenzyme S, G100-purified exoenzyme S, and purified exotoxin A were performed on a Beckman 120 amino acid analyzer (Beckman Instruments, Toronto, Ontario) according to the directions of the manufacturer.

Analysis of the reaction product. Radiolabeled products formed in the ADP-ribosyl transferase assays were examined by SDS-PAGE. The radiolabeled products formed from 10 μl of purified exoenzyme S (2 mg/ml, 80 U of enzyme activity per mg of protein) and 10 μl of purified exotoxin A (1 mg/ml, 100 U of enzyme activity per mg of protein) were examined in separate assay mixtures. The enzymatic activity of exotoxin A was potentiated by first incubating the exotoxin A in 4 M urea–1% dithiothreitol for 15 min at 15°C (10). Trichloroacetic acid precipitates collected from ADP-ribosyl transferase assay mixtures were washed three times with 5% trichloroacetic acid, suspended in SDS-PAGE sample buffer as described above, and electrophoresed in 10% gels (14). Samples contained approximately 100 μg of protein, all of which was electrophoresed. After electrophoresis, the gel was dried under reduced pressure and heat onto paper backing and exposed to X-ray film (AR 50; Eastman Kodak Co., Rochester, N.Y.) for 30 days for autoradiography.

TABLE 1. Purification of exoenzyme S

Sample	Total protein (mg)[a]	Specific enzyme activity (U/mg of protein)[b]	Recovery of enzyme activity (%)	Specific toxic activity (LD_{50}/mg of protein)[c]	Recovery of toxic activity (%)
Culture supernatant[d]	1,880.0	22.0	100.0	0.1	100.0
$(NH_4)_2SO_4$ precipitate	141.0	29.6	10.4	1.5	112.0
DEAE					
Pool 1	3.0	1,000.0	7.5	28.0	44.7
Pool 2	4.5	333.0	3.8	<0.001	0
Acetone precipitate	2.2	256.0	1.4	38.0	44.5
G-100 eluate	1.1	80.0	0.2	125.0	73.1

[a] Protein concentrations were measured by the dye-binding assay (9).
[b] Enzyme activity was measured by the ADP-ribosyl transferase assay (8).
[c] Toxic activity was measured in the mouse lethality bioassay.
[d] Cultures (5 liters) were grown for 36 h at 32°C in S medium.

RESULTS

Purification. The procedure for purification of exoenzyme S consists of four major steps: ammonium sulfate precipitation, anion-exchange chromatography on DEAE-Sephacel, acetone precipitation in the presence of 1 M NaCl, and G-100 Superfine gel filtration chromatography. Exoenzyme S was monitored during purification by assaying ADP-ribosyl transferase activity, mouse toxicity, and SDS-PAGE. Details of yield and specific activity for a typical preparation are shown in Table 1. The yields were calculated by arbitrarily designating the culture supernatant material as 100%.

Precipitation of culture supernatant with 60% ammonium sulfate resulted in an approximate 25% increase in specific enzyme activity (counts per minute per milligram of protein) as measured by the ADP-ribosyl transferase assay. More significantly, ammonium sulfate precipitation of culture supernatant resulted in a 15-fold increase in specific toxic activity (LD_{50} per milligram of protein) for mice. This trend was seen throughout the purification procedure when increases in specific enzyme activity lagged behind increases in specific toxic activity.

After ammonium sulfate precipitation and dialysis of the precipitate overnight at 4°C, this material was subjected to DEAE-Sephacel ion-exchange chromatography. The profile obtained from the ion-exchange column after a 0 to 1 M NaCl gradient elution is shown in Fig. 1. Two major absorbance peaks were obtained. Fractions from these were pooled and designated pool 1 (0.4 to 0.5 M NaCl) and pool 2 (0.5 to 0.7 M NaCl). When these pools were assayed for enzyme activity and toxic activity, pool 2 was shown to have no toxic activity for mice. Thus, the remainder of the purification procedure was performed with pool 1 material, which demonstrated a 45-fold increase in specific enzyme activity and a

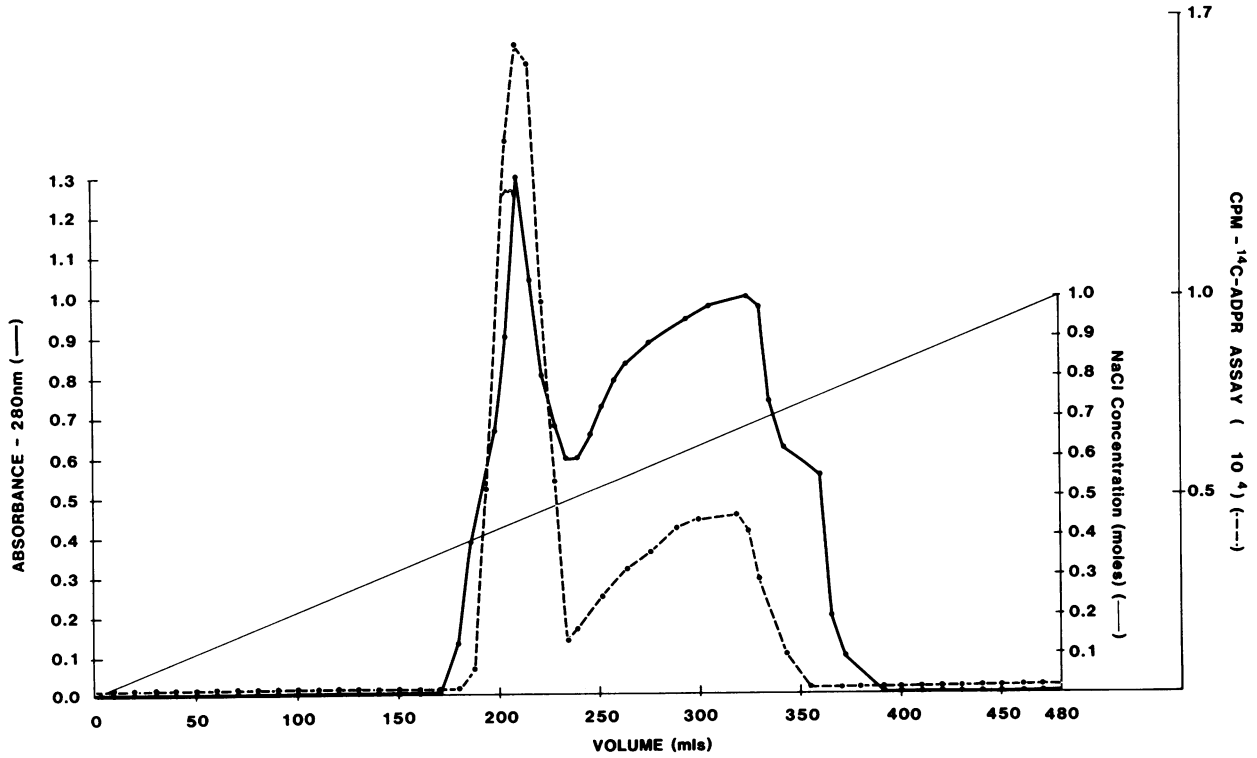

FIG. 1. Ion-exchange chromatography (DEAE-Sephacel) of ammonium sulfate-precipitated *P. aeruginosa* DG1 culture supernatant. Dialyzed precipitate was applied to a column (2.6 by 30 cm) equilibrated in Tris buffer (50 mM, pH 7, containing 0.01 M NaCl). Elution was accomplished with 500 ml of 0.01 to 1.01 M NaCl gradient. Elution was followed by measurement of the A_{280}. ADPR, ADP-ribosyl.

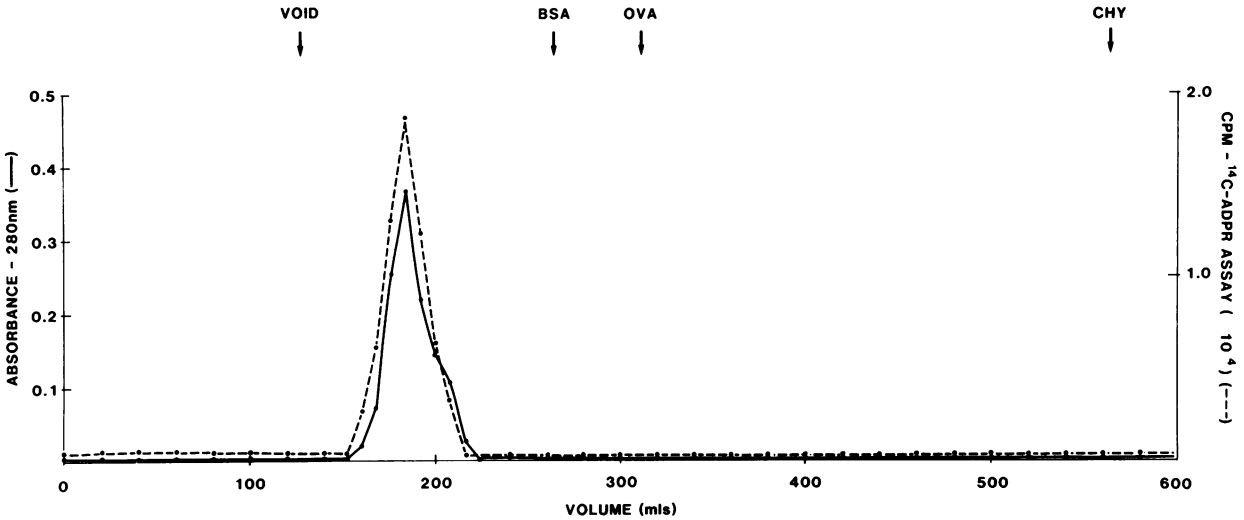

FIG. 2. Gel filtration chromatography (G-100 Superfine) of acetone-precipitated exoenzyme S. Dialyzed precipitate was applied to a column (2.6 by 77.5 cm) equilibrated in Tris buffer (50 mM, pH 7.6). Elution was accomplished by rinsing with 600 ml of equilibrating buffer and was monitored by measuring the A_{280}. Elution volumes of molecular weight markers are indicated by arrows. BSA, M_r = 66,000; OVA, ovalbumin, M_r = 45,000; CHY, chymotrypsin, M_r = 25,000. ADPR, ADP-ribosyl.

280-fold increase in specific toxic activity over the culture supernatant starting material.

The pool 1 material was adjusted to an approximate final NaCl concentration of 1.0 M and precipitated with acetone at 0°C. This resulted in material with 12-fold-higher specific enzyme activity and 380-fold-higher specific toxic activity than the starting material. The material resulting from the acetone precipitation step had significantly increased toxic activity for mice, but lower specific enzyme activity than the pool 1 material obtained from ion-exchange chromatography.

This same observation was made on material obtained from the final step in the purification procedure, gel filtration chromatography. The acetone-precipitated material was dialyzed extensively and placed on a G-100 Superfine gel filtration column. The elution profile (Fig. 2) showed a single absorbance peak at an elution volume of approximately 180 ml. This corresponded to an approximate M_r of 105,000 as estimated by the procedure of Andrews (1), in which the elution volume of the material was compared with those of several proteins with known molecular weights. The elution volumes of BSA (M_r = 66,000), ovalbumin (M_r = 45,000), and chymotrypsin (M_r = 25,000) were determined under conditions similar to those used in the purification of exoenzyme S. The material obtained in this final purification step was 4-fold more enzymatically active and 1,250-fold more toxic than the original starting material.

Figure 3 represents a silver-stained SDS-PAGE profile of material eluted from the G-100 column along with molecular weight markers for comparison. A 50-μg sample of G-100 eluate material was loaded onto lane 1. A major protein band corresponding to M_r = 50,000 was seen along with minor bands of M_r = 64,000, M_r = 45,000, and M_r = 20,000. Each of these protein bands was examined for ADP-ribosyl transferase activity after elution from unstained gels. The specific activity varied considerably among these. Gel slices (1 mm thick) were incubated for 12 h at 4°C in 0.1 ml of buffer (0.05 M Tris hydrochloride [pH 7.0], 1 mM EDTA, 50 mM dithiothreitol) after repeated injection through an 18-gauge needle. The eluted proteins were then concentrated by lyophilization and suspended in 10 μl of distilled water. These samples were then assayed for ADP-ribosyl transferase activity as previously described. The approximate protein concentration in each of the gel slices was calculated on the basis of the percentage of total protein loaded onto the gel. These percentages were obtained from densitometric tracings of a corresponding stained gel. The specific activities (units per milligram of protein) for the four protein bands are: M_r = 64,000, 1.2; M_r = 50,000, 16.6; M_r = 45,000, 0; and M_r = 20,000, 0.3. In each case, those proteins which reacted with wheat germ extract after elution for SDS-PAGE also reacted with purified oubain-sensitive ATPase (Sigma). This indicates that a single ADP-ribosyl transferase was present in the original material.

Properties of purified exoenzyme S. Spectral analysis of purified exoenzyme S demonstrated a single symmetrical

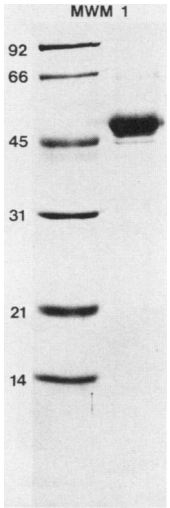

FIG. 3. SDS-PAGE analysis (silver stained, heated, and reduced) of material eluted from G-100 column along with molecular weight markers. Lane MWM, Phosphorylase *b* (M_r = 92,000 [92], BSA (M_r = 66,000), ovalbumin (M_r = 45,000), carbonic anhydrase (M_r = 31,000), soybean trypsin inhibitor (M_r = 21,000), and lysozyme (M_r = 14,000). Lane 1, 50 μg of G-100 eluate material.

TABLE 2. Cytotoxic activity of exoenzyme S and exotoxin A

Cell line	TCD_{50}[a] (ng)	
	Exoenzyme S	Exotoxin A
CHO	1.5×10^3	0.5
Vero	10.0	1.0
HeLa	5.0	0.8
L	5.0	1.0

[a] 50% tissue culture dose (TCD_{50}) is the amount of toxin required to kill 50% of the 2×10^4 cells in a microtiter well at 48 h.

absorbance peak at 278 nm indicating the presence of protein and the absence of any detectable pigment. The absence of contaminating nucleic acid was shown by an A_{280}/A_{260} ratio of 1. The absence of contaminating endotoxin was shown by the inability to demonstrate a positive limulus assay (Difco Laboratories; Detroit, Mich.) on the purified material. Further, the purified exoenzyme S contained no contaminating exotoxin A as demonstrated by a negative reaction in immunoblot analysis with high-titered rabbit antiserum to exotoxin A. Each of the above assays was performed on purified exoenzyme S concentrated to 2 mg/ml with a specific activity of 80 U/mg of protein and a mouse LD_{50} of 8 μg.

The toxicity of purified exoenzyme S for mice was measured in a mouse lethality bioassay and found to have an LD_{50} of 8 μg as compared with a value of 0.3 μg obtained for exotoxin A (20-g mice, intraperitoneal injections). The toxicity of exoenzyme S for a variety of tissue culture cell lines was also measured. These data, along with those obtained with exotoxin A, are presented in Table 2. As in the mouse lethality bioassay, exotoxin A was significantly more toxic than exoenzyme S for each of the cell lines tested.

An investigation of the radiolabeled reaction products formed in wheat germ extract in the presence of [adenine-^{14}C]NAD^+ and exotoxin A or exoenzyme S confirmed the previously reported results of Iglewski et al. (11). The principal product, labeled in the presence of exotoxin A had an M_r of 100,000 (the known molecular weight of EF-2) (Fig. 4). Additionally, products of lower molecular weight were also labeled, albeit to a much lower extent. Presumably, these could be breakdown products of the M_r = 100,000 labeled product. Three major reaction products were noted in the presence of purified exoenzyme S (Fig. 4). These corresponded to approximately M_r = 50,000, M_r = 30,000, and M_r = 25,000. These same reaction products were labeled in the presence of crude exoenzyme S (DEAE eluate material; data not shown).

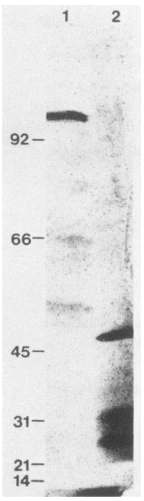

FIG. 4. Autoradiogram of SDS-PAGE displaying radiolabeled reaction products formed in ADP-ribosyl transferase assay. Molecular weight markers as described in the legend to Fig. 3. Lanes: 1, reaction products labeled in wheat germ extract in the presence of exotoxin A and [adenine-^{14}C]NAD^+; 2, reaction products labeled in wheat germ extract in the presence of exoenzyme S and [adenine-^{14}C]NAD^+.

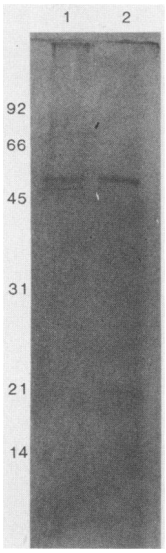

FIG. 5. Immunoblot analysis of monoclonal antibodies reacted with *P. aeruginosa* DG1 culture supernatant. Molecular weight markers are described in the legend to Fig. 3. Lane 1, MCA1; lane 2, MCA2. Procedures used for the immunoblot analysis are described in the text.

Anti-exoenzyme S monoclonal antibodies. Monoclonal antibodies were prepared against exoenzyme S and divided into two classes, depending on their reactivity with the different species of exoenzyme S seen on SDS-PAGE. Figure 5 shows an immunoblot analysis of representatives of the two classes of monoclonal antibodies, designated MCA1 and MCA2, reacted with *P. aeruginosa* DG1 culture super-

TABLE 3. Partial amino acid composition comparison of exotoxin A with exoenzyme S[a]

Amino acid	Exotoxin A[b]	G-100-purified exoenzyme S[b]	Affinity-purified exoenzyme S[b]
Asp	11.5	12.4	12.3
Thr	3.0	6.9	6.9
Ser	5.2	6.7	6.7
Glu	12.3	9.0	8.9
Pro	7.1	3.2	3.4
Gly	10.0	10.7	10.2
Ala	11.6	15.4	15.9
Val	6.6	7.4	6.8
Met	0.8	0.6	0.8
Ile	4.0	5.4	5.7
Leu	11.1	10.1	10.3
Tyr	3.6	2.7	2.7
Phe	1.8	4.0	3.8
His	2.4	0.9	1.0
Lys	2.9	5.8	5.8
Arg	7.0	5.1	5.0

[a] Performed on a Beckman 120 amino acid analyzer.
[b] Expressed as percentage of total protein.

natant. MCA1 reacts with the M_r = 50,000 and the M_r = 45,000 components of exoenzyme S, while MCA2 reacts with the M_r = 64,000 (weak), M_r = 50,000, and M_r = 20,000 species. These two monoclonal antibodies were analyzed further with respect to their ability to neutralize exoenzyme S enzyme activity as well as exoenzyme S cytotoxicity for HeLa cells. ELISA titers, and immunoglobulin class. MCA1 is an IgM monoclonal antibody, has an ELISA titer against exoenzyme S of 1:43,648, has an enzyme neutralization titer of only 1:2, and has a cytotoxicity neutralization titer of 1:10,912. MCA is an IgG monoclonal antibody, has an ELISA titer against exoenzyme S of 1:43,648, has an enzyme neutralization titer of 1:10,192, and has a cytotoxicity neutralization titer of 1:2,048.

MCA2 was used for affinity purification of exoenzyme S from the acetone-precipitated DEAE-Sephacel eluate. Extremely poor yields were obtained from the monoclonal antibody affinity column procedure. From 2 mg of starting material, 10 μg of affinity-purified exoenzyme S was obtained. This material demonstrated no activity in the ADP-ribosyl transferase assay; however, it was extremely toxic for mice (LD_{50} of 1 μg). The silver-stained SDS-PAGE profile of material eluted from the affinity column was identical to that seen in Fig. 3.

Amino acid composition. Partial amino acid compositional analyses of affinity-purified exoenzyme S. G-100-purified exoenzyme S, and exotoxin A are presented in Table 3. These analyses were performed three times with separately purified material with identical results. No significant differences in amino acid composition were noted between the two exoenzyme S preparations. When compared with exotoxin A, purified exoenzyme S contained higher amounts of glutamic acid, proline, leucine, tyrosine, and arginine and lower amounts of aspartic acid, threonine, serine, alanine, valine, isoleucine, phenylalanine, and lysine. Thus, significant amino acid compositional differences exist between the two toxins.

DISCUSSION

Iglewski and co-workers (17–19) have purified exoenzyme S in a form which is nontoxic to experimental animals or isolated tissue culture cells. In the work described herein, we purified exoenzyme S by conventional means, as well as by affinity chromatography employing monoclonal antibody to exoenzyme S, in a form which is toxic for animals and a variety of tissue culture cell lines.

The critical step in the presently described purification procedure for exoenzyme S was the use of organic solvent extraction in the presence of high salt concentration. Previous attempts to purify exoenzyme S have employed detergent to solubilize the protein, a step which most likely has detoxified the molecule (17). The use of acetone-high NaCl to solubilize the enzyme appears either to deaggregate the enzyme or to dissociate it from some high-molecular-weight molecule. This then allows the use of conventional gel chromatography techniques to purify the enzyme, whereas previously exoenzyme S has eluted in the void volume of all gel chromatographic columns employed (23).

It is interesting to note that while the purified exoenzyme S is considerably more toxic than the starting material, the specific enzymatic activity of the purified enzyme is only slightly higher than that of the original starting culture supernatant. It seems plausible that, similar to *P. aeruginosa* exotoxin A and other bacterial toxins (16), exoenzyme S is purified in a nonenzymatically active or proenzyme form. In the case of exotoxin A, the enzyme may be converted to the active form by incubation with urea and dithiothreitol (10). To date, we have not been able to activate exoenzyme S in vitro, although a variety of procedures used for other toxins have been employed including urea-dithiothreitol, trypsin-dithiothreitol (7), and various heat treatments (100°C for 2 min; 60°C for 5 min).

The estimated M_r of exoenzyme S obtained by gel filtration chromatography was 105,000. It is probable that the different molecular weight species of exoenzyme S seen on SDS-PAGE are forms of the same enzyme as evidenced by their enzymatic activity or reactivity, or both with monoclonal antibodies and that the M_r = 105,000 form eluted from the G-100 column corresponds to aggregates of the different species. All the different species react with monoclonal antibody directed against exoenzyme S, and three of the four species are enzymatically active in the ADP-ribosyl transferase assay. It seems reasonable to propose that exoenzyme S is composed of various subunits, as has been shown for a number of bacterial toxins (16), and that the different SDS-PAGE protein species are the subunit components of the toxin. Alternatively, those different protein species could be degradation products owing to proteolysis; however, this is not supported by the observation that incorporation of EDTA or phenylmethylsulfonyl fluoride or both, known protease inhibitors, does not change the SDS-PAGE profile of the purified material. Structure-function studies employing our expanding library of monoclonal antibodies directed against exoenzyme S should enable us to address this question.

In comparison studies with exotoxin A, exoenzyme S was shown to be immunologically and biochemically distinct from exotoxin A. The amino acid composition and the reaction product analysis studies indicate that these are undoubtedly two distinct enzymes. Antiserum to exotoxin A dose not neutralize exoenzyme S activity, nor does it react with exoenzyme S in either ELISA or immunoblot assay. We showed that exotoxin A is more toxic to mice and to tissue culture cell lines including CHO, L. Vero, and HeLa cells than is exoenzyme S. Studies to determine the toxicity of exoenzyme S for other animals and additional cell lines are in progress.

The procedure described for the purification of exoenzyme S will enable us to address a number of important questions including the structure-function relationships between exotoxin A and exoenzyme S and the intriguing question as to why an organism such as *P. aeruginosa* would produce two separate and distinct ADP-ribosyl transferases.

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