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Purification and Characterization of Exoenzyme S from *Pseudomonas aeruginosa* 388

SCOTT M. KULICH, DARA W. FRANK, AND JOSEPH T. BARBIERI*

Department of Microbiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226

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Exoenzyme S was purified >1,500-fold from the culture supernatant fluid of *Pseudomonas aeruginosa* 388 at high yield without utilization of solvents or detergents. Two proteins, with apparent molecular sizes of 53 and 49 kDa, cofractionated with exoenzyme S activity. Rabbit anti-49-kDa-protein immunoglobulin G was prepared by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis-purified 49-kDa protein as immunogen. Anti-49-kDa-protein IgG inhibited the ADP-ribosyltransferase activity of purified exoenzyme S in a dose-dependent manner, which indicated a role for the 49-kDa protein in the ADP-ribosylation reaction. Analysis by ultrafiltration showed that exoenzyme S activity and the 53- and 49-kDa proteins cofractionated and that exoenzyme S was apparently >300 kDa in size. Urea (8 M) and 1.0% Triton X-100 reversibly decreased the apparent molecular sizes of exoenzyme S activity and the 53- and 49-kDa proteins to between 30 and 100 kDa.

Conditions such as neutropenia, burn wounds, and cystic fibrosis are predisposing factors to *Pseudomonas aeruginosa* infections (2). *P. aeruginosa* produces both extracellular and cell-associated products which contribute to its virulence (21). Two of these virulence factors, exotoxin A (11) and exoenzyme S (5, 12), catalyze the covalent transfer of the ADP-ribose portion of NAD to eukaryotic target proteins. Exotoxin A and exoenzyme S differ with respect to heat stability (12), eukaryotic target protein specificity (6, 8, 11, 12), amino acid residue that is ADP-ribosylated (8, 13), and requirement of a eukaryotic accessory protein termed factor activating exoenzyme S (FAS) for in vitro expression of exoenzyme S activity (7). This last property makes exoenzyme S similar to cholera toxin, which requires a eukaryotic accessory protein termed ARF for the full expression of ADP-ribosyltransferase activity (10, 14).

In vivo, exoenzyme S has been implicated as a virulence factor required by *P. aeruginosa* for dissemination from burn wounds (18, 22) and tissue destruction in patients with chronic lung infections (19, 22, 30). To date, the physiological eukaryotic target protein(s) of exoenzyme S has not been resolved. In vitro, exoenzyme S ADP-ribosylates several eukaryotic target proteins, including vimentin (6) and $p21^{c\text{-}H\text{-}ras}$ (8). Two *Pseudomonas* proteins have been associated with the expression of exoenzyme S activity. Nicas and Iglewski (20) isolated a 53-kDa enzymatically inactive form and a 49-kDa enzymatically active form of exoenzyme S. The 49-kDa form of exoenzyme S was subsequently characterized by Coburn et al. (7).

Our studies have focused on the biochemical analysis of exoenzyme S as an ADP-ribosyltransferase. In this report, we describe the purification of exoenzyme S from the culture supernatant fluid of *P. aeruginosa* 388, show that the 53- and 49-kDa proteins cofractionate with exoenzyme S activity, demonstrate that anti-49-kDa-protein immunoglobulin G (IgG) inhibits the ADP-ribosyltransferase activity of exoenzyme S, and show that exoenzyme S activity and the 53- and 49-kDa proteins exist as a noncovalent aggregate that can be deaggregated with 8 M urea and 1.0% Triton X-100.

* Corresponding author.

MATERIALS AND METHODS

Materials. [*Adenylate* ^{32}P-*phosphate*]NAD was purchased from DuPont-New England Nuclear. A model 12 stirred cell and ultrafiltration membranes were purchased from Amicon (W. R. Grace & Co.). ^{125}I was purchased from Amersham. ^{125}I-protein A was prepared by the chloramine-T method (23). Densitometry was performed with an AMBIS system. The bovine serum albumin (BSA) standard used in protein and densitometry quantitation was purchased from Pierce.

Bacterial strains and culture conditions. *P. aeruginosa* 388 and 388 *exs1*::Tn*1* have been described previously (12, 20). *P. aeruginosa* strains were stored in 10% Difco skim milk at −70°C and plated on VBM medium (27) at 37°C for 48 h prior to broth culturing. For cultivation of *P. aeruginosa* 388 for exoenzyme S purification, eight colonies were inoculated into 200 ml of deferrated (Biorad Chelex 4000) Trypticase soy broth supplemented with 1% glycerol, 0.1 M monosodium glutamate, and 0.01 M nitrilotriacetic acid. This medium was hydrated in NANOpure (Barnstead) water and termed TSBD (20). Cultures were shaken vigorously at 32°C for 18 h. The final A_{540} (1-cm path length) of the culture was >4.0. For cultivation of *P. aeruginosa* 388 and 388 *exs1*::Tn*1* for analysis of anti-49-kDa-protein IgG specificity, several colonies were inoculated into 10 ml of TSBD. Cultures were shaken vigorously at 32°C for 13 h. The final A_{540} (1-cm path length) of the culture was between 5 and 6.

Purification of exoenzyme S. All purification steps were performed at 4°C. The culture fluid was centrifuged at 10,000 × *g* for 10 min and then at 25,000 × *g* for 30 min. The soluble culture fluid was brought to a final concentration of 55% ammonium sulfate (1.2 ml of saturated ammonium sulfate was added per ml of culture fluid). After 3 h, the ammonium sulfate-precipitable material was collected by centrifugation at 25,000 × *g* for 40 min, suspended in 20 ml of 25 mM Tris-HCl (pH 7.6) containing 2 M urea (Tris–2 M urea), and dialyzed (molecular size cutoff, 6 to 8 kDa) against Tris–2 M urea. The conductivity of the dialyzed material was less than

that of Tris–2 M urea containing 50 mM NaCl, which was empirically determined to allow exoenzyme S activity to bind to a DEAE matrix. The dialyzed material was chromatographed on DEAE-Sephacel (20 ml of resin equilibrated in Tris–2 M urea) with a 160-ml linear gradient of 0 to 250 mM NaCl at a flow rate of 1 ml/min; 6-min fractions were collected. Fractions containing peak exoenzyme S activity were pooled (conductivity of the pool was equivalent to that of Tris–2 M urea containing 75 mM NaCl) and subjected to ultrafiltration with a XM300 membrane. As originally designated by Iglewski and coworkers (12), ADP-ribosyltransferase activity enriched for during this purification will be termed purified exoenzyme S. Proteins were reduced with β-mercaptoethanol and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel (10% monomer) electrophoresis (PAGE) as described previously (16). Proteins were visualized by Coomassie brilliant blue R-250 staining or Western blot (immunoblot) analysis (3) using 2 nM anti-49-kDa-protein IgG as primary antibody (preparation of this antibody is described below) followed by autoradiography after incubation with ^{125}I-protein A. Nitrocellulose was stained with Ponceau S prior to probing with antisera to allow alignment of proteins on the nitrocellulose with signals on the autoradiogram.

Deaggregation of purified exoenzyme S with urea and Triton X-100. The stability of the purified exoenzyme S aggregate was measured in the presence of several combinations of urea and Triton X-100. All incubations were performed at room temperature in a model 12 stirred cell. Purified exoenzyme S (1.0 ml) was diluted to 10 ml with 25 mM Tris-HCl (pH 7.6) containing 8 M urea (Tris–8 M urea) and incubated for 14 h. Urea-treated exoenzyme S was subjected to ultrafiltration with an XM300 membrane. The resulting ultrafiltration retentate (approximately 0.5 ml) was diluted to 10 ml with Tris–8 M urea and immediately subjected to XM300 ultrafiltration. Sequentially, the resulting ultrafiltration retentate was diluted into 10 ml of Tris–8 M urea containing 0.01, 0.1, and then 1.0% (wt/vol) Triton X-100; incubated for 1 h under constant stirring; and subjected to XM300 ultrafiltration. The resulting retentates were washed once with buffer at each Triton X-100 incubation. The final XM300 retentate and all ultrafiltrates were assayed for exoenzyme S activity. The majority of exoenzyme S was present in the XM300 ultrafiltrate treated with Tris–8 M urea–1.0% Triton X-100. This XM300 ultrafiltrate was termed deaggregated exoenzyme S.

Reaggregration of exoenzyme S. Deaggregated exoenzyme S was sequentially subjected to ultrafiltration with YM100 and YM30 membranes. Following this serial ultrafiltration, the majority of exoenzyme S was found in the YM30 retentate.

This YM30 retentate was subjected to DEAE chromatography (3 ml of resin equilibrated in Tris–2 M urea) to separate the free Triton X-100 from exoenzyme S. Exoenzyme S bound to the DEAE resin and was eluted in Tris–2 M urea with a step gradient of 50 to 250 mM NaCl (50 mM increments of NaCl) and then with Tris–2 M urea containing 500 mM NaCl. Fractions containing exoenzyme S were pooled and subjected to XM300 ultrafiltration at 4°C. All steps preceding XM300 ultrafiltration were performed at room temperature. The resulting ultrafiltrate and retentate were assayed for exoenzyme S.

Generation of anti-49-kDa-protein IgG. Antisera to the 49-kDa protein were prepared by a protocol described previously (15). Purified exoenzyme S was subjected to reduced SDS-PAGE (10% acrylamide gel) and electroblotted to nitrocellulose. The nitrocellulose was stained with Ponceau S (Sigma Chemical Co.), and the 49-kDa protein was excised, suspended in 500 μl of dimethyl sulfoxide, and used for intradermal immunization of rabbits. Rabbits were immunized at 3-week intervals. Approximately 1 week after each immunization, sera were collected. IgG was purified from sera of pre- and postimmunized rabbits with an ImmunoPure IgG Purification kit from Pierce.

(i) Specificity of anti-49-kDa-protein IgG. *P. aeruginosa* cells (2×10^9) of strains 388 and 388 *exs1*::Tn*1* were centrifuged at 14,000 × *g* for 10 min at 4°C, and the culture supernatant fluid and cells were treated as follows. Culture supernatant fluid (640 μl) was brought to a final concentration of 55% ammonium sulfate and incubated on ice. After 2 h, the ammonium sulfate-precipitable material was collected by centrifugation at 12,000 × *g* for 15 min at 4°C, suspended in 26 μl of SDS sample buffer containing β-mercaptoethanol, and boiled for 5 min. Samples were normalized for cell number and subjected to reduced SDS-PAGE (11% polyacrylamide gel) in triplicate. One gel was stained with Coomassie blue, and two gels were subjected to Western blot analysis as described above by using preimmune IgG and anti-49-kDa-protein IgG. Cell pellets were washed twice with ice-cold phosphate-buffered saline (PBS), suspended in 300 μl of SDS sample buffer containing β-mercaptoethanol, boiled for 5 min, and centrifuged at 12,000 × *g* for 2 min at 4°C to pellet the insoluble material. Samples were normalized for cell number, subjected to reduced SDS-PAGE (11% polyacrylamide gel) in triplicate, and analyzed for total protein and reactivity with anti-49-kDa-protein IgG as described above.

(ii) Inhibition of ADP-ribosylation activity of exoenzyme S by anti-49-kDa-protein IgG. Inhibition of ADP-ribosylation activity of exoenzyme S by anti-49-kDa-protein IgG was measured as described below.

Determination of exoenzyme S activity. Two assays were used to measure exoenzyme S ADP-ribosyltransferase activity.

(i) ADP-ribosylation of proteins in wheat germ extract (20). Reaction mixtures contained (in a final volume of 27 μl) 0.2 M sodium acetate (pH 6.0), 1 μM [*adenylate* ^{32}P-*phosphate*] NAD (specific activity, 6 Ci/mmol), 40 μg of wheat germ extract (4), and enzyme. In this assay, the wheat germ extract served as the source of both FAS and target proteins for ADP-ribosylation. To normalize the protein concentration in the reaction mixture, enzyme preparations assayed for exoenzyme S were diluted in Tris–2 M urea containing 0.1 mg of chicken egg albumin per ml. Reaction mixtures were incubated at room temperature and at timed intervals were spotted onto Whatman 3MM filters impregnated with 10% trichloroacetic acid (TCA). Filters were washed with 7.5% TCA (four 30-min washes), rinsed with methanol, and dried. Incorporation of radiolabel into TCA-precipitated material was determined by scintillation counting.

(ii) ADP-ribosylation of SBTI (7). Reaction mixtures contained (in a final volume of 40 μl) 0.2 M sodium acetate (pH 6.0), 30 μM soybean trypsin inhibitor (SBTI), and 30 μM [*adenylate* ^{32}P-*phosphate*]NAD (specific activity, 0.1 Ci/mmol), 2 μg of wheat germ extract, and enzyme. In this assay, the wheat germ extract served as a source of FAS. At timed intervals, reaction mixtures were added to SDS sample buffer containing β-mercaptoethanol and boiled for 5 min. Following reduced SDS-PAGE (13.5% polyacrylamide gel), incorporation of radiolabel into SBTI was analyzed by autoradiography and quantitated by scintillation counting of the SBTI protein. Data were expressed as moles of ADP-

TABLE 1. Purification of exoenzyme S from culture medium of *P. aeruginosa* 388[a]

Stage	Total protein (mg)[b]	Activity (U)[c]	Sp act (U/mg)	% Activity recovered[d]
Supernatant fluid				
10,000 × *g*	3,280	17,300	5.26	100
25,000 × *g*	3,280	20,200	6.17	117
55% ammonium sulfate precipitation				
Predialysis	37.2	28,200	758	163
Postdialysis	31.6	25,000	791	145
DEAE chromatography	2.96	15,200	1,760	88
Ultrafiltration (XM300 retentate[e])	2.34	18,900	8,090	110

[a] Data are from a single representative purification of exoenzyme S from a culture of *P. aeruginosa* 388 grown in TSBD medium.
[b] Calculated by using A_{280} and a BSA standard.
[c] One unit of activity equals 1 pmol of NAD incorporated into TCA-precipitable wheat germ extract protein per min.
[d] 100% activity = 17,300 U.
[e] The XM300 retentate was termed purified exoenzyme S.

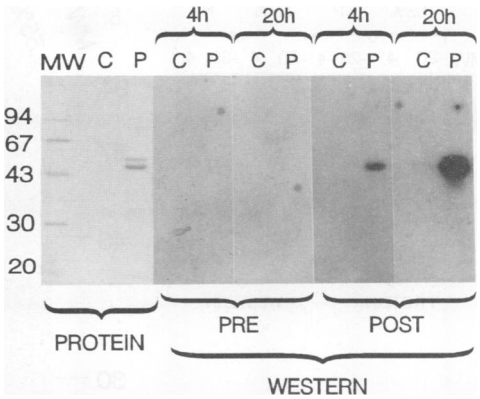

FIG. 1. Purification of exoenzyme S from culture medium of *P. aeruginosa* 388. Total protein (2.5 μg) from the 25,000 × *g* supernatant fluid (lanes C) and purified exoenzyme S (lanes P) was subjected to reduced SDS-PAGE (10% polyacrylamide gel). Gels were stained for protein with Coomassie brilliant blue R-250 (PROTEIN) or transferred to nitrocellulose (WESTERN) and probed with either preimmune IgG (PRE) or anti-49-kDa-protein IgG (POST). Autoradiograms of 4- and 20-h exposures are shown. MW indicates Coomassie-stained protein standards with molecular sizes in kilodaltons.

ribosylated SBTI per minute per mole of 49-kDa protein. The molar concentration of 49-kDa protein was extrapolated densitometrically from a Coomassie-stained reduced SDS–10% polyacrylamide gel by using a BSA standard and assuming equal molar staining of BSA and the 49-kDa protein.

In experiments which measured the effects of preimmune IgG and anti-49-kDa-protein IgG on exoenzyme S activity, purified exoenzyme S was incubated with an equal volume of IgG or PBS at room temperature for 30 min prior to analysis of ADP-ribosyltransferase activity. Threefold dilutions of IgG were made in PBS containing 0.25 mg of BSA per ml.

Amino acid composition and amino-terminal amino acid sequencing of the 49-kDa protein. Purified exoenzyme S was subjected to 10% reduced SDS-PAGE and electroblotted to a polyvinylidene difluoride membrane. Following amido black staining, the 49-kDa band was excised and subjected to amino acid composition analysis and amino-terminal sequencing as described previously (1).

Protein concentration determination. Protein concentrations of exoenzyme S were calculated by measurement of A_{280}, with BSA as a standard. Rabbit IgG concentration was calculated by using 13.5 A_{280} units as the molar extinction coefficient in a 1-cm cell (25).

RESULTS

Purification of exoenzyme S. The ADP-ribosyltransferase activity of exoenzyme S was purified from the culture supernatant fluid of *P. aeruginosa* 388 by utilizing ammonium sulfate precipitation, DEAE chromatography, and XM300 ultrafiltration. This purification protocol yielded a >1,500-fold increase in specific activity and essentially complete recovery of ADP-ribosyltransferase activity (Table 1). As originally designated by Iglewski and coworkers (12), ADP-ribosyltransferase activity enriched for during this purification is termed purified exoenzyme S. Tris–2 M urea was included in all buffers used for purification subsequent to ammonium sulfate precipitation. During the development of this protocol, we observed that the addition of 2 or 4 M urea to the resuspended ammonium sulfate precipitate increased the yield of exoenzyme S as determined by Western blotting (this antibody against exoenzyme S is described in reference 20) and that exoenzyme S was not inhibited following preincubation with up to 6 M urea (data not shown).

Total protein (2.5 μg) from the 25,000 × *g* supernatant fluid and purified exoenzyme S of *P. aeruginosa* 388 was subjected to SDS-PAGE followed by Coomassie staining and Western blotting using anti-49-kDa-protein IgG (preparation of the IgG fraction is described in Materials and Methods). No prominent proteins were visible in the Coomassie-stained gel of the 25,000 × *g* supernatant fluid (Fig. 1, lane C), and only upon overexposure on the Western blot was the 49-kDa protein detected. In contrast, purified exoenzyme S was enriched for two prominent Coomassie-stained proteins with apparent molecular sizes of 53 and 49 kDa (Fig. 1, lane P), and the 49-kDa protein and, to a lesser extent, the 53-kDa protein were detected in the short exposure of the Western blot. The molecular sizes of the two proteins which copurified with exoenzyme S activity agree with previously reported molecular sizes of the enzymatically inactive (53-kDa) and enzymatically active (49-kDa) forms of exoenzyme S (20). This purification protocol also enriched for several lower-molecular-weight proteins; most notable were proteins possessing apparent molecular sizes of 35, 31, and 19 kDa (Fig. 1, lane P).

Although DEAE chromatography provided only a twofold enrichment in the specific activity of exoenzyme S (Table 1), it was included in the purification protocol for two reasons. First, DEAE chromatography separated exoenzyme S from a protease activity which reduced exoenzyme S activity and degraded the 53- and 49-kDa proteins (Fig. 2A). This observation is discussed below. Second, DEAE chromatography separated exoenzyme S activity from a 50-kDa protein contaminant (50-kDa protein is indicated by an arrow in Fig. 2B). Elimination of this 50-kDa protein from preparations of purified exoenzyme S activity was essential, since the 49-kDa protein that was used as an immunogen, for amino acid analysis, and for amino-terminal protein sequencing was obtained from SDS-polyacrylamide gels, where the 49- and

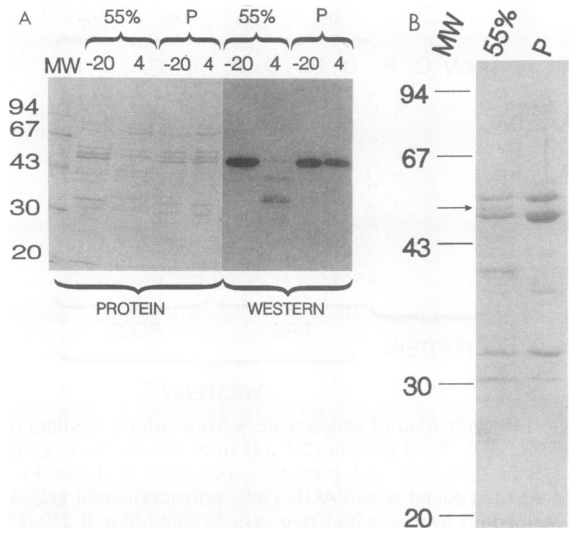

FIG. 2. DEAE chromatography during purification protocol decreases degradation of exoenzyme S and removes 50-kDa protein. (A) Exoenzyme S (25 U) from a 55% ammonium sulfate precipitate of the 25,000 × *g* supernatant fluid suspended in Tris–2 M urea (55%) and purified exoenzyme S (P) were incubated at either −20°C or 4°C. After 48 h, samples were assayed for ADP-ribosyltransferase activity and subjected to reduced SDS-PAGE (10% polyacrylamide gel). Gels were stained for protein with Coomassie blue (PROTEIN) or transferred to nitrocellulose (WESTERN) and probed with anti-49-kDa-protein IgG. An autoradiogram of a 15-h exposure of the Western blot is shown. (B) Exoenzyme S (100 U) from a 55% ammonium sulfate precipitate of the 25,000 × *g* supernatant fluid resuspended in Tris–2 M urea (55%) and purified exoenzyme S (P) were subjected to reduced SDS-PAGE (10% polyacrylamide gel). Gels were stained for protein with Coomassie blue. Arrow denotes the position of the 50-kDa contaminant. MW indicates Coomassie-stained protein standards with molecular sizes in kilodaltons.

50-kDa proteins possessed similar relative migrations (Fig. 2B).

Amino acid composition and amino-terminal amino acid sequence of the 49-kDa protein. The amino acid composition of the 49-kDa protein appears to be acidic, possessing 23.5% Asx and Glx and 8.4% Arg and Lys (Table 2). This is in agreement with data from isoelectric focusing of purified exoenzyme S (15a) as well as the previously reported acidic nature of exoenzyme S (pI of exoenzyme S was between 4.4 and 4.5) (26). According to two determinations, the 20 amino-terminal amino acids of the 49-kDa protein were Met-His-Ile-Gln-Ser-Leu-Gln-Gln-Ser-Pro-Ser-Phe-Ala-Val-Glu-Leu-His-Gln-Ala-Ala. This sequence is identical to one determined by independent observations by J. Lile and B. Iglewski (17a) for all 20 residues and by J. Coburn and D. M. Gill (7a) for the first 7 residues.

Properties of anti-49-kDa-protein IgG. (i) Specificity of anti-49-kDa-protein IgG. Although the 53- and 49-kDa proteins copurify with exoenzyme S activity, it is difficult to prove by strictly biochemical techniques that a specific protein and not a contaminating protein is responsible for an enzymatic activity. Therefore, immunological methods were used to investigate the role of the 49-kDa protein in the expression of exoenzyme S activity. The 49-kDa protein was used as an immunogen in rabbits, and IgG was purified from pre- and postimmune sera. Postimmune IgG (anti-49-kDa-protein IgG) recognized the 49-kDa protein on Western blots of a concentrated culture supernatant fluid of *P. aeruginosa* 388 and purified exoenzyme S (Fig. 3, WESTERN, POST). Anti-49-kDa-protein IgG did not react with the cell lysate of *P. aeruginosa* 388, which suggests that cell-associated steady-state levels of 49-kDa protein were low. Anti-49-kDa-protein IgG did not react with the concentrated supernatant fluid or cell lysates of *P. aeruginosa* 388 *exs1*::Tn*1*, an isogenic mutant of 388 which is deficient in production of exoenzyme S activity and 53- and 49-kDa proteins (20). Preimmune IgG did not show a specific reaction to proteins in concentrated supernatant fluid or cell lysates of *P. aeruginosa* 388 or 388 *exs1*::Tn*1* or to purified exoenzyme S (Fig. 3, WESTERN, PRE).

Anti-49-kDa-protein IgG also reacted to the 53-kDa protein both in the concentrated supernatant fluid of *P. aeruginosa* 388 and in the purified exoenzyme S but with a lower

TABLE 2. Partial amino acid composition of 49-kDa protein

Amino acid residue	Mol% in 49-kDa protein[a]
Asx	6.5
Thr	2.9
Ser	7.1
Glx	17.0
Pro	0.4
Gly	12.9
Ala	13.3
Val	7.1
Met	2.2
Ile	4.0
Leu	13.5
Tyr	1.0
Phe	2.0
Lys	2.9
His	1.6
Arg	5.5

[a] The 49-kDa protein (43 pmol) was subjected to acid hydrolysis followed by amino acid composition determination. Analysis was performed with the Beckman model 6300 amino acid analyzer. Molar percentages of amino acids were based on a molecular size estimation of 49 kDa and an average amino acid molecular size of 100 Da.

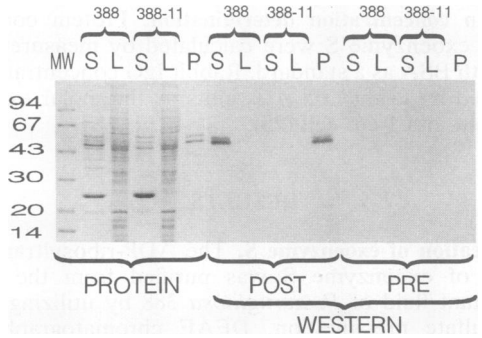

FIG. 3. Specificity of anti-49-kDa-protein IgG. Cell lysates (lanes L) and 55% ammonium sulfate precipitates of culture supernatant fluids (lanes S) were collected from *P. aeruginosa* 388 (lanes 388), 388 *exs1*::Tn*1* (lanes 388-11), and 2.5 μg of purified exoenzyme S (lanes P) and subjected to reduced SDS-PAGE (11% polyacrylamide gel). Samples of supernatant fluid and lysates were normalized for cell number prior to electrophoresis. Gels were stained for protein with Coomassie blue (PROTEIN) or transferred to nitrocellulose (WESTERN) and probed with either anti-49-kDa-protein IgG (POST) or preimmune IgG (PRE). Autoradiograms of 4-h exposures are shown. MW indicates Coomassie-stained protein standards with molecular sizes in kilodaltons.

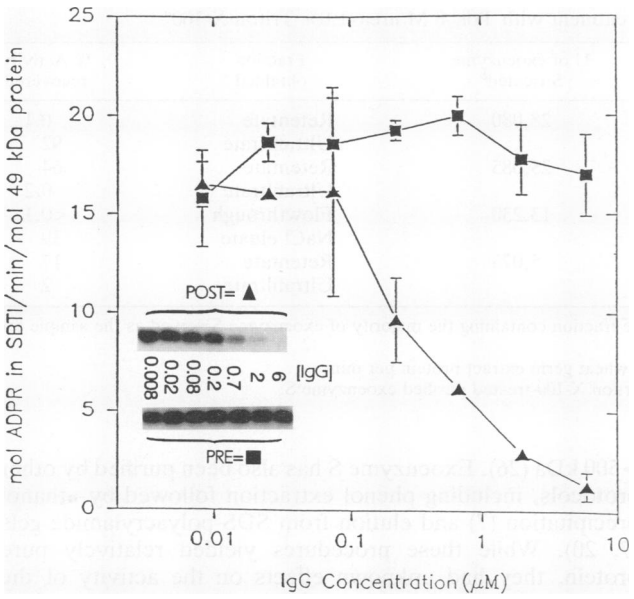

FIG. 4. Inhibition of exoenzyme S by anti-49-kDa-protein IgG. Purified exoenzyme S was preincubated for 30 min at room temperature with either preimmune IgG (PRE) or anti-49-kDa-protein IgG (POST). Reaction mixtures were assayed in duplicate for ADP-ribosyltransferase activity. Datum points show mean incorporation of ^{32}P label from NAD into SBTI (error bars indicate standard errors of the means) in a 60-min incubation. In the absence of IgG, incorporation of ^{32}P from [*adenylate* ^{32}P-*phosphate*]NAD into SBTI equaled 20.7 mol of ADP-ribosylated SBTI per min per mol of 49-kDa protein. The insert is an autoradiogram from a reduced SDS–13.5% polyacrylamide gel showing the incorporation of ^{32}P into SBTI from one set of datum points. Micromolar concentration of IgG in the preincubation reaction is indicated. ADPR, ADP ribose.

immunological signal than Coomassie-stained protein with respect to the 49-kDa protein (Fig. 1 and 3). The absolute reactivity of anti-49-kDa-protein IgG with the 53-kDa protein varied with the blocking protein utilized in the Western blot. In Fig. 1, the Western blot was blocked with 1% hemoglobin, while the Western blot shown in Fig. 3 was blocked with 1% nonfat dry milk. The reason for this differential reactivity is not clear.

(ii) Inhibition of ADP-ribosylating activity of exoenzyme S by anti-49-kDa-protein IgG. Anti-49-kDa-protein IgG inhibited the ADP-ribosylation of SBTI by purified exoenzyme S (Fig. 4, POST). In a linear velocity reaction, anti-49-kDa-protein IgG showed a dose-dependent inhibition of the ADP-ribosylating activity of purified exoenzyme S, with the highest concentration of IgG yielding essentially complete inhibition. In contrast, preimmune IgG did not inhibit the ADP-ribosyltransferase activity of purified exoenzyme S (Fig. 4, PRE).

We also examined the ability of the anti-49-kDa-protein IgG to inhibit the ADP-ribosylation of a second set of target proteins by a crude preparation of exoenzyme S. Anti-49-kDa-protein IgG inhibited ADP-ribosylation of wheat germ extract proteins by a 10,000 × *g* supernatant fluid of *P. aeruginosa* 388. This inhibition occurred in a dose-dependent manner with respect to anti-49-kDa-protein IgG. Preimmune IgG did not inhibit the incorporation of ADP-ribose into wheat germ extract proteins by this crude source of enzyme (data not shown). These data show that anti-49-kDa-protein IgG neutralizes both crude and purified preparations of exoenzyme S.

Proteolysis of 53- and 49-kDa proteins and loss of exoenzyme S activity in the 55% ammonium sulfate-precipitated supernatant fluid. The 53- and 49-kDa proteins were selectively degraded during incubation of the 55% ammonium sulfate-precipitable fraction of the 25,000 × *g* supernatant fluid of *P. aeruginosa* 388 at 4°C for 48 h (Fig. 2A). Western blot analysis of this extract confirmed the degradation of the anti-49-kDa-protein IgG reactive proteins (Fig. 2A). Measurement of ADP-ribosyltransferase activity also showed that storage of the 55% ammonium sulfate-precipitable fraction at 4°C resulted in a loss of approximately 90% of exoenzyme S activity relative to its activity prior to storage. The selective degradation of the 53- and 49-kDa proteins and loss of exoenzyme S activity support the model that either the 53- and/or the 49-kDa protein is required for exoenzyme S activity. In contrast, both the 53- and 49-kDa proteins and exoenzyme S activity were stable during the incubation of purified exoenzyme S under the same conditions (Fig. 2A). Both the 53- and 49-kDa proteins and exoenzyme S activity in either the 55% ammonium sulfate-precipitable fraction of the 25,000 × *g* supernatant fluid or purified exoenzyme S were stable following storage at −20°C.

Analysis of the exoenzyme S aggregate. (i) Properties of the exoenzyme S aggregate. Several observations showed that exoenzyme S activity and the 53- and 49-kDa proteins copurified as a soluble aggregate. First, in three independent experiments, 60, 75, and 95% of the exoenzyme S activity in the 25,000 × *g* supernatant fluid was soluble after ultracentrifugation in excess of 100,000 × *g* for 1 h. Second, exoenzyme S activity, as well as the 53- and 49-kDa proteins, in the supernatant fluid following ultracentrifugation eluted in the void volume of a Sephacryl-S200 gel filtration column when chromatographed in 25 mM Tris-HCl (pH 7.6) with or without 6 M urea. Third, the 53- and 49-kDa proteins and exoenzyme S activity from a partially purified preparation of exoenzyme S activity eluted in the void volume of a Sephacryl-S300 gel filtration column (molecular size exclusion for globular proteins equals 1,500 kDa) when chromatographed in Tris–2 M urea with or without 0.01% (wt/vol) Triton X-100 (data not shown).

(ii) Deaggregation of exoenzyme S. The ability of combinations of urea and Triton X-100 to deaggregate purified exoenzyme S was measured by assaying the conversion of the aggregated exoenzyme S to a form which filtered through an XM300 membrane. In a serial experiment performed in Tris–8 M urea, neither 0.01 nor 0.1% Triton X-100 deaggregated exoenzyme S, but 1.0% Triton X-100 converted exoenzyme S to a form which filtered through the XM300 membrane (data not shown). The Tris–8 M urea–1.0% Triton X-100-treated exoenzyme S in the XM300 ultrafiltrate also filtered through a YM100 membrane but was retained on a YM30 membrane (Table 3). These results showed that 8 M urea–1.0% Triton X-100-treated exoenzyme S had an apparent molecular size of between 30 and 100 kDa.

(iii) Reaggregration of exoenzyme S. The reversibility of the aggregation of exoenzyme S was also determined (Table 3). Tris–8 M urea–1.0% Triton X-100-treated exoenzyme S was chromatographed on DEAE-Sephacel to separate free Triton X-100 from exoenzyme S and to equilibrate exoenzyme S in Tris–2 M urea. Exoenzyme S eluted from the DEAE column with NaCl. Exoenzyme S in the NaCl eluate was retained on an XM300 membrane. These results indicated that the deaggregated exoenzyme S reaggregated upon removal of Tris–8 M urea–1.0% Triton X-100.

TABLE 3. Reaggregration of exoenzyme S following treatment with Tris–8 M urea–1.0% Triton X-100[a]

Sample	Treatment	U of exoenzyme S treated[b]	Fraction obtained	% Activity recovered
Deaggregated exoenzyme S[c]	YM100 ultrafiltration	28,080	Retentate	0.1
			Ultrafiltrate	92
YM100 ultrafiltrate	YM30 ultrafiltration	25,085	Retentate	64
			Ultrafiltrate	0.2
YM30 retentate	DEAE chromatography	13,230	Flowthrough	<0.1
			NaCl eluate	39
DEAE NaCl eluate	XM300 ultrafiltration	5,073	Retentate	17
			Ultrafiltrate	2

[a] Data were generated from a serially performed experiment. In this protocol, the fraction containing the majority of exoenzyme S served as the sample for the next treatment.
[b] One unit of activity equals 1 pmol of NAD incorporated into TCA-precipitable wheat germ extract protein per min.
[c] Deaggregated exoenzyme S is the XM300 ultrafiltrate of Tris–8 M urea–1.0% Triton X-100-treated purified exoenzyme S.

SDS-PAGE analysis of the products of the deaggregation and reaggregation of the exoenzyme S showed that (i) the 53- and 49-kDa proteins cofractionated with exoenzyme S activity; (ii) some degradation of anti-49-kDa-protein IgG reactive material had occurred; and (iii) following this treatment, the 49-kDa protein possessed a decreased electrophoretic mobility, while the mobility of the 53-kDa band did not appear to change (Fig. 5). This shift in electrophoretic mobility may be due to the binding of Triton X-100 to the 49-kDa protein.

DISCUSSION

In this study, exoenzyme S activity was purified >1,500-fold from the culture medium of *P. aeruginosa* 388. Woods and Que have also reported a purification protocol for exoenzyme S which led to a >1,000-fold increase in specific toxic activity in mice and a 4-fold increase in exoenzyme S specific activity (29). Both protocols employed ammonium sulfate precipitation and DEAE chromatography in the initial purification steps. The purification protocol described by Woods and Que (29) used 33% acetone precipitation in 1 M NaCl and gel filtration to purify exoenzyme S. Our protocol utilized DEAE chromatography and then XM300 ultrafiltration, which took advantage of an earlier report that exoenzyme S activity possessed an apparent molecular size of >500 kDa (26). Exoenzyme S has also been purified by other protocols, including phenol extraction followed by ethanol precipitation (7) and elution from SDS-polyacrylamide gels (7, 20). While these procedures yielded relatively pure protein, they had unknown effects on the activity of the enzyme. These investigators did not report the fold increase in specific activity of exoenzyme S activity.

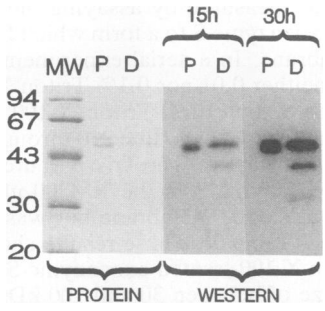

FIG. 5. SDS-PAGE of purified exoenzyme S before and after treatment with Tris–8 M urea–1% Triton X-100. Exoenzyme S ADP-ribosyltransferase activity (25 U) from purified exoenzyme S (lanes P) and the XM300 retentate of the DEAE-Sephacel pool of the deaggregated exoenzyme S (lanes D) were subjected to reduced SDS-PAGE (10% polyacrylamide gel). Gels were stained for protein with Coomassie blue (PROTEIN) or transferred to nitrocellulose (WESTERN) and probed with anti-49-kDa-protein IgG. Autoradiograms of 15- and 30-h exposures are shown. MW indicates Coomassie-stained protein standards with molecular sizes in kilodaltons.

Our data agree with those from earlier studies (26), which described exoenzyme S activity as a high-molecular-weight aggregate. Physically, exoenzyme S appears to be similar to a vacuolating toxin released by *Helicobacter pylori* which exists as an aggregate of >970 kDa (9). Also, exoenzyme S and *H. pylori* vacuolating toxin appear to exert similar cytopathic effects, stimulating the intracellular vacuolation of susceptible eukaryotic cells (17, 28). Perhaps these two proteins are members of a family of bacterial products which exert their effects via a common final pathway which leads to cellular vacuolization. Biochemical characterization of these bacterial products will determine whether a relationship exists.

Two proteins with apparent molecular sizes of 53 and 49 kDa, as analyzed by reduced SDS-PAGE, copurified with exoenzyme S activity. These data agreed with data from several earlier studies which (i) implicated a 49-kDa protein as the enzymatically active form of exoenzyme S and a 53-kDa protein as the enzymatically inactive form of exoenzyme S (20), (ii) reported exoenzyme S activity associated with a 49-kDa protein following elution from SDS-PAGE (7, 20), and (iii) showed coinduction of the 53- and 49-kDa proteins and exoenzyme S activity in supernatant fluids of *P. aeruginosa* 388 culture by the chelating agent nitrilotriacetic acid (9a). Two sets of data for our study provide additional evidence that either the 53- or the 49-kDa protein or both play a role in the expression of exoenzyme S activity. First, anti-49-kDa-protein IgG inhibited exoenzyme S activity. Second, a *Pseudomonas* protease coordinately degraded the 53- and 49-kDa proteins and reduced exoenzyme S activity in a crude extract.

Anti-49-kDa-protein IgG generated in this study should prove to be a useful reagent. Anti-49-kDa-protein IgG inhibited exoenzyme S-mediated ADP-ribosylation of both a specific target, SBTI, and multiple proteins in a wheat germ extract. Also, anti-49-kDa-protein IgG neutralized exoenzyme S activity both in a crude extract containing exoenzyme S activity and in purified exoenzyme S.

Western blot analysis demonstrated that anti-49-kDa-protein IgG cross-reacted with the 53-kDa protein. It is not clear whether this cross-reactivity represents shared epitopes

between the 53- and 49-kDa proteins or whether the 49-kDa protein used as an immunogen contained some 53-kDa protein. Earlier studies have reported immunological cross-reactivity between the 53- and 49-kDa forms of exoenzyme S (20) and have shown that 53- and 49-kDa proteins generate similar tryptic peptides (22), evidence that the 53- and 49-kDa proteins are related. The precise molecular role of the 53- and 49-kDa proteins in the expression of exoenzyme S activity and the relationship between these two proteins are currently under investigation.

The amino-terminal amino acids of the 49-kDa protein purified by our protocol and by those of Iglewski and coworkers (17a) and Gill and Coburn (7a) were identical. Sokol et al. (24) have cloned an adhesive form of exoenzyme S which, when expressed in *Escherichia coli*, yielded a 68-kDa protein which did not possess detectable ADP-ribosyltransferase activity; the deduced amino acid sequence of this clone was not reported. Future experimentation will seek to define the relationship between the 53- and 49-kDa proteins and the adhesive form of exoenzyme S.

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