Fermentation, Purification, and Characterization of Protective Antigen from a Recombinant, Avirulent Strain of Bacillus anthracis

J. W. FARCHAUS,* W. J. RIBOT, S. JENDREK, AND S. F. LITTLE

Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21702-5011

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Bacillus anthracis, the etiologic agent for anthrax, produces two bipartite, AB-type exotoxins, edema toxin and lethal toxin. The B subunit of both exotoxins is an M₆, 83,000 protein termed protective antigen (PA). The human anthrax vaccine currently licensed for use in the United States consists primarily of this protein adsorbed onto aluminum oxyhydroxide. This report describes the production of PA from a recombinant, asporogenic, nontoxigenic, and nonencapsulated host strain of B. anthracis and the subsequent purification and characterization of the protein product. Fermentation in a high-tryptone, high-yeast-extract medium under nonlimiting aeration produced 20 to 30 mg of secreted PA per liter. Secreted protease activity under these fermentation conditions was low and was inhibited more than 95% by the addition of EDTA. A purity of 85 to 95% was achieved for PA by dialfiltration and anion-exchange chromatography, while greater than 95% final purity was achieved with an additional hydrophobic interaction chromatography step. The purity of the PA product was characterized by reversed-phase high-pressure liquid chromatography, sodium dodecyl sulfate (SDS)-capillary electrophoresis, capillary isoelectric focusing, native gel electrophoresis, and SDS-polyacrylamide gel electrophoresis. The biological activity of the PA, when combined with excess lethal factor in the macrophage cell lysis assay, was comparable to previously reported values.

The gram-positive organism Bacillus anthracis, the etiologic agent of anthrax, is the only member of the genus Bacillus capable of causing epidemic disease in humans and other mammals. B. anthracis grows in long chains and is nonmotile; virulent strains harbor two endogenous plasmids, pXO1 (29, 43) and pXO2 (10, 46), which code for the major known virulence factors of this organism. Plasmid pXO2 harbors the genes responsible for the synthesis of the glutamyl polypeptide capsule, which gives the strains their characteristic mucoid appearance in the presence of bicarbonate (10, 24, 25). Plasmid pXO1 harbors the structural genes for toxin production and regulation (30, 39–42, 48). Toxigenic B. anthracis strains secrete two bipartite exotoxins, lethal toxin and edema toxin. The secreted M₆, 83,000 protein, known as protective antigen (PA), serves as the B component of both toxins (20). It binds to an unidentified receptor on the cell surface, where it is cleaved by cellular protease(s) to an M₆, 63,000 form that exposes the binding site for the A components lethal factor (LF) and edema factor (EF), which bind competitively (4, 9, 17).

It was discovered during the late 1800s and early 1900s that cultures of virulent B. anthracis could be attenuated by growth at 42 to 43°C. The attenuation observed with such Pasteur-type vaccine strains resulted from the loss of plasmid pXO1. Fully virulent pXO2+ pXO1+ strains were thus attenuated by conversion to the pXO2+ pXO1− genotype. Other attenuated strains, such as the Sterne strain, spontaneously lost pXO2 while retaining pXO1. Culturing the Sterne strain at 42°C resulted in the loss of pXO1 and produced the avirulent pXO1− pXO2− strain referred to as ΔSterne-1 (11).

The currently licensed human vaccine is produced by growing the pXO1+ pXO2− V770-NP1-R strain of B. anthracis in minimal medium in the presence of bicarbonate under microaerophilic conditions and adsorbing the sterile filtered culture supernatant to aluminum oxyhydroxide adjuvant (36, 37). The protective component of the vaccine appears to be PA. Although the vaccine has proven efficacious (7, 13, 14, 37), the current vaccine strain has several limitations, including a sporogenic and fully toxigenic genotype. Production of vaccine from this strain results in lot-to-lot variability due to inconsistent PA production levels, inclusion of undefined PA proteolytic degradation products, and inclusion of other bacterial products including LF and EF (31).

To eliminate these limitations, an avirulent, nontoxigenic strain, ΔSterne-1, was selected as a host for PA expression. The recombinant plasmid pPA102 was created by subcloning a 6-kb BamHI fragment harboring the PA structural gene and flanking sequence originally cloned from the endogenous B. anthracis plasmid pXO1 (15). The 6-kb fragment was inserted into the gram-positive vector pUB110 and transformed into B. subtilis 168, and PA-positive transformants were selected (15). Subsequent characterization of the B. subtilis transformants revealed that spontaneous deletions had occurred, resulting in the loss of substantial portions of the original 6-kb insert, including the bicarbonate regulation (42) of PA production. A stable kanamycin-resistant, PA-positive version of the plasmid was isolated and termed pPA102 (15). This plasmid was electrottransformed into B. anthracis ΔSterne-1 to specifically restore constitutive PA production (12). Subsequently, an asporogenic variant was selected and characterized (49). We describe here the fermentation, purification, and characterization of recombinant PA produced from ΔSterne-1(pPA102)CR4.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The B. anthracis pXO1− pXO2− ΔSterne-1 strain used in this study (11) was electrottransformed with pPA102.
was calibrated after 16 h of polarization by setting the DO2 zero value to 0 nA (45), except that no calcium was added other than the amount present in the pore-size cellulose acetate filters (Millipore Corp.). The sterile supernatant was dry cell weight per 10 ml of culture with respect to OD600 revealed that the oxygen in response to the current measured DO2 value during the fermentation was calibrated between pH 7 and 10 before sterilization by using standard buffers and Milli-Q (MQ) (Millipore Corp., Marlborough, Mass.) water was added as necessary during the fermentation. Unless otherwise specified, chemicals were obtained from Sigma (St. Louis, Mo.).

Fermentation conditions. The fermentations were performed with a Micro I top-drive fermentor (New Brunswick Scientific, New Brunswick, N.J.) with a 20-liter-working-volume 316-L stainless steel vessel equipped with two Rushton impellers whose diameter was equal to one-third the vessel diameter. The lower impeller was positioned on the drive shaft at a distance equal to the impeller diameter from the bottom of vessel, while the remaining impeller was positioned 1.5 times the impeller diameter above the lower impeller. The fermentor was also equipped with a ML1400 process controller, a two-gas mixer, and Advanced Fermentation software on some (New Brunswick Scientific). The vessel and medium were sterilized by exposure to 121°C for 15 min. The short sterilization cycle was required to minimize Millard-type and other medium degradation reactions. The total elapsed time at temperatures in excess of 37°C was less than 45 min. Subsequent testing revealed that medium stability was maintained for more than 45 h under growth conditions.

The polarographic dissolved oxygen (DO2) probe (Ingold, Wilmington, Pa.) was calibrated after 16 h of polarization by setting the DO2 zero value to 0 nA with a DO2-pH simulator (Valley Instrument Co., Esoton, Pa.). The 100% value was set to the oxygen tension at 37°C in the medium after aeration with air (1 vol/vol/min) at an agitation rate of 150 rpm for 6 to 7 h. The entire 200-ml subculture was then inoculated into 30 ml of medium in a 4-liter baffled Erlenmeyer flask. This seed culture was incubated at 37°C with shaking at 150 rpm for 6 to 7 h until a maximum optical density of 600 nm (OD600) between 1.5 and 3.5 was attained. The 5% (vol/vol) seed culture volume was transferred aseptically to the 20-L fermentor. The initial OD600 was recorded, and a sample of the inoculum was streaked on sheep blood agar plates and incubated at 37°C to verify inoculum purity. OD600 values were maintained at 75% of saturation during the fermentations by increasing the agitation from the initial 250 rpm to a maximum of 500 rpm. Once the rpm maximum was achieved, the two-gas mixer supplemented the process with air and oxygen. The oxygen saturation at 1 vol/vol was 98%, the mixture rate and percentages of air and pure oxygen were controlled by the two-gas mixer, with the relative proportions of air and oxygen being governed by an AFS (New Brunswick) algorithm which increased or decreased the percentage of oxygen required to maintain the current measured fermentation. Both gases had a working pressure of approximately 22 lb/in2.

The cell density and PA production analysis were carried out by manually sampling the fermentation liquor through a sterile sampling port. Analysis of the dry cell weight per 10 ml of culture with respect to OD600 revealed that the relationship between the two parameters was linear, confirming that OD600 accurately measured cell density. We measured OD600 after diluting the culture with sterile medium to yield an OD600 value of less than 0.2.

Activity-protein determinations. Protease activity in culture supernatants was determined with resorufin-labeled casein (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Cultures were grown in baffled shake flasks with shaking at 150 rpm at 37°C until the late log phase or early stationary phase. The cells were removed by sterile filtration of the culture with low-protein-binding 0.22-μm pore-size cellulose acetate filters (Millipore Corp.). The sterile supernatant was used immediately in the assays, which were performed as described by Twining (45), except that no calcium was added other than the amount present in the medium. Protease activity was measured spectroscopically at 574 nm.

Assay of the M. 83,000 PA in crude fermentation liquor by SDS-PAGE. Samples for PA determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were filtered through 0.22-μm pore-size filters and stored at −20°C. An additional 2 mL of supernatant was applied to the gel. The samples were later concentrated approximately 10-fold with Centricron 30 concentrators (Amicon, Beverly, Mass.) by centrifugation at 4,000 × g and were desalted twice by diluting to the original volume with 20 ml of HEPES buffer (pH 7.3) containing 0.05 M Tris, 0.05 M EDTA, and repeating the desalting process three times. At −20°C, the samples were reconstituted with the same volume of 100 mM ammonium acetate buffer (pH 7.0) and stored at −70°C.

When greater than 90% final purity was required, the PA was applied at 22 to 23°C to the QE-purified product, frozen under nitrogen, and stored at −70°C.
Analysis of purified M<sub>r</sub> 83,000 PA. The purity of the PA was assessed by several different methods. SDS-PAGE was performed as described above, except that the samples were not lyophilized. Native PAGE was performed as recommended by the manufacturer, with 4 to 15% acrylamide Phast gels (Pharmacia Biotech, Piscataway, N.J.).

Reversed-phase HPLC was performed at 22 to 23°C with an R1/M C<sub>4</sub> column (Perceptive Biosystems). The column was equilibrated in 90% solvent A (0.05% [wt/vol] NaOH)–10% solvent B (80% [vol/vol] acetonitrile, 0.05% [wt/vol] NaOH) before injection of 50- to 100-μg samples. The column was developed at a linear flow rate of 1.800 cm/h with a linear 15-column-volume gradient to 80% solvent B. The absorbance at 280 nm (A<sub>280</sub>) was monitored.

SDS-capyillary electrophoresis (CE) was performed with a 270A-HT CE system interfaced with Turbochrom version 4.1 software (Perkin-Elmer Applied Biosystems, Foster City, Calif.) and ProSort SDS-protein analysis reagent (Perkin-Elmer Applied Biosystems) in a 42-cm by 55-μm (inner diameter) fused-silica capillary. Capillary conditioning and equilibration with ProSort reagent were carried out as described by the manufacturer. PA samples (3 to 4 mg/ml) were diluted 1:1 with 10 mM HEPEs (pH 7.3) and then further diluted with SDS–2-mercaptoethanol buffers as suggested by the manufacturer.

Automated N-terminal sequencing was performed approximately 100 pmol of PA purified by ion-exchange chromatography or by reversed-phase HPLC. All the protein samples were transferred into neutral buffers and further desalted with PD10 desalting columns (Bio-Rad) equilibrated with 5 mM NaCl and 1 mM CaCl<sub>2</sub>, before being applied to a Perkin-Elmer Applied Biosystems 470A Sequenator (Perkin-Elmer). Phenylthiohydantoin-derived amino acids were identified with a model 120A phenylthiohydantoin analyzer from the same manufacturer. C-terminal sequencing was carried out on polyvinylidene difluoride-adsorbed PA with a Perkin-Elmer Applied Biosystems 477 Sequenator by using the allylation approach.

The biological activity of purified PA was assessed by an in vitro cytotoxicity test (6). Briefly, various concentrations of PA were combined with 40 ng of LF per ml and added to J774A.1 macrophage monolayers in a volume of 0.1 ml. After 4 h of incubation, 25 μl of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Boehringer Mannheim) at 5 mg/ml was added per well. The cells were lysed after 2 h, and 15F7 (22), each at a 1:2,000 dilution in PBS-M containing 0.05% (vol/vol) Tween 20. After the membranes were washed with PBS-M–0.05% (vol/vol) Tween 20, immunoreactive bands were detected either with the enhanced chemiluminescence (ECL) reagent as recommended by the manufacturer (Pierce, Rockford, Ill.) or by adding 4-chloro-1-naphthol and hydrogen peroxide.

RESULTS

Medium effects and growth. The first step in the development of the PA production method was to assess the effects of growth medium on bacterial cell growth, PA production, and protease activity. Good growth rates and high biomass were achieved previously with the ΔSterne-1 strain in a high-tryp-tone, high-yeast-extract medium (5). When the same medium was investigated with the ΔSterne-1(pPA102)CR4 strain under conditions of low or high aeration in shake flask experiments, the highest cell densities and PA production levels were achieved under aerobic conditions. PA production levels were compared in late-log- to early-stationary-phase aerobic cultures in complete medium (Fig. 1, lane 1), in medium with 25% of the total yeast extract found in complete medium (lane 2), and in medium with 25% of the total yeast found in complete medium (Fig. 1, lane 3). The largest amount of intact M<sub>r</sub> 83,000 PA was observed in complete medium (lane 1), while smaller amounts were generated with the reduced-yeast-extract medium shown in lane 2 and little, if any, intact M<sub>r</sub> 83,000 PA was found in the reduced-yeast-extract medium (lane 3). However, the cell growth in all three media was comparable to the growth in the complete culture (lane 1), reaching 7 OD<sub>600</sub> units, while the reduced-yeast-extract (lane 2) and reduced-yeast-extract (lane 3) cultures reached 10 and 8 OD<sub>600</sub> units, respectively.

We also determined the protease activity in complete medium and 25% trypthone medium culture supernatants by the resorufin-labeled casein assay. Fourfold-higher protease activity was measured for the reduced-tryptone cultures when mid-log-phase cultures of 5 to 6 OD<sub>600</sub> units were compared (Table 1). The activity in the reduced-tryptone medium increased to as much as 50-fold greater than in complete medium when cultures in stationary phase were compared (data not shown). A second addition of tryptone to the culture supernatants after

**TABLE 1. Determination of protease activity in culture supernatants and reduction of measurable activity by trypthone and EDTA**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Additive</th>
<th>Protease activity (μU/OD&lt;sub&gt;600&lt;/sub&gt; unit) (mean ± SD)</th>
<th>% Reduction in measurable activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>None</td>
<td>15.4 ± 0.6</td>
<td>46</td>
</tr>
<tr>
<td>Complete</td>
<td>Tryptone</td>
<td>8.3 ± 0.4</td>
<td>96</td>
</tr>
<tr>
<td>Complete</td>
<td>EDTA</td>
<td>0.5 ± 0.1</td>
<td>96</td>
</tr>
<tr>
<td>1/4 trypthone</td>
<td>None</td>
<td>56.9 ± 2.4</td>
<td>56</td>
</tr>
<tr>
<td>1/4 trypthone</td>
<td>Tryptone</td>
<td>24.8 ± 1.0</td>
<td>96</td>
</tr>
<tr>
<td>1/4 trypthone</td>
<td>EDTA</td>
<td>0.8 ± 0.1</td>
<td>96</td>
</tr>
</tbody>
</table>

* Aliquots of each culture were sterile filtered and immediately used for protease assays. Activity was measured by the resorufin-labeled casein assay at 574 nm after a 16-h incubation at 32°C with 12.5 units of trypthone/μl (0.1 μU/OD<sub>600</sub> unit). The trypthone added was prepared as a 10-fold-concentrated stock (330 g/liter) in MQ water and filtered through a 0.2-μm-pore-size filter to remove insoluble debris. The amount of trypthone added was equivalent to the amount in complete medium (33 g/liter). The final concentration of EDTA (pH 8.0) was 24 mM.
The bacteria were removed by sterile filtration; reduced the measurable effect of the protease on the resorufin-labeled casein in both complete and reduced tryptone medium by 46% and 56%, respectively (Table 1). From the comparable total cell growth in the two media and these protease activity results, the higher levels of tryptone optimized PA production by acting not only as a substrate for growth but also as a surrogate protease substrate, which slowed the proteolytic degradation of PA. Inhibitory studies revealed that the protease activity released into the medium was more than 95% inhibited by the addition of EDTA (Table 1). The combination of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) with EDTA was found to be no more inhibitory than EDTA alone (data not shown). These observations were confirmed by SDS-PAGE analysis of culture supernatants spiked with purified PA (data not shown).

**Fermentation.** Figure 2 shows the pH, DO₂, percent O₂, and agitation values for a representative fermentation at the 20-liter level. The pH was monitored but not controlled during the course of the fermentation. A small drop in pH can be seen between elapsed fermentation times (EFT) of 50 and 175 min, which we attribute to the production of organic acids from the metabolism of carbohydrates supplied with the yeast extract. After an approximate EFT of 190 to 200 min, the pH began to increase, consistent with the release of ammonium from the aerobic metabolism of amino acids and peptides. After the pH had increased by 0.01 unit, a second addition of tryptone was made as a 10-fold concentrate of the amount added initially to ensure that tryptone never became limiting during the fermentation. Once the tryptone had been added, the pH decreased transiently at 200 to 205 min due to minor temperature and pH differences between the added tryptone and the vessel. The pH increased to a maximum of 8.5 during the course of the fermentation but remained within the acceptable range of 6 to 9 for *B. anthracis* ΔSterne-1(pPA102)CR4 (data not shown).

The DO₂ value dropped from the initial 100% to the set point of 75% at an EFT of ca. 75 min (Fig. 2). The 75% set point was maintained by increasing the agitation from 250 rpm to a maximum of 500 rpm. As shown in Fig. 2, the maximum agitation was reached within 175 min. Since further increases in agitation were counterproductive, due to increased cell shear, supplementing the process air with 100% O₂ gas was necessary because attempts to control the DO₂ by increasing agitation and/or pressure alone failed to maintain a DO₂ value above zero throughout the fermentation. As seen in Fig. 2, oxygen effectively maintained the DO₂ set point once the maximum agitation was achieved. Oxygen supplementation increased steadily throughout the log phase of growth until the deceleration (early stationary) phase was reached at 9 to 12 OD₆₀₀ units and a constant decrease in the percent oxygen occurred. This decrease is seen in Fig. 2 at an EFT of 300 to 325 min. The percentage of oxygen decreased steadily as the cells entered stationary phase, with the exception of a transient rise at 350 min. Once the oxygen supplementation reached zero, the agitation also decreased, suggesting very little demand for oxygen.

The growth curves and yield of *Mₚ* 83,000 PA as a function of EFT are shown in Fig. 3 for two representative fermentations. The data demonstrate the reproducibility of the fermentation process and suggest that the maximum cell density under the conditions used is 14 to 15 OD₆₀₀ units. The doubling time was 53.4 ± 3.8 min, and the specific growth rate was 0.0130 ± 0.0009 min⁻¹. The growth curves confirmed that the decrease in oxygen consumption at an EFT of 300 to 325 min shown in Fig. 2 occurred during the deceleration (late log to early stationary) phase of growth. The plot of *Mₚ* 83,000 PA against EFT also demonstrated that the yield of *Mₚ* 83,000 PA also reached a maximum during the deceleration phase of growth. More importantly, it also clearly showed the subsequent decline in product attributable to the protease activity released into the medium by the host strain.

Even though the growth data under nonlimiting aeration proved extremely reproducible, the yield of *Mₚ* 83,000 PA was less consistent late in the fermentation (Fig. 3). Based on this yield variability and the rather sudden onset of product loss due to degradation, we selected the decrease in oxygen supplementation seen in Fig. 2 as the main criterion for determining the onset of the deceleration phase and terminating the fermentation. This allowed harvesting at a point near the max-

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**FIG. 2.** Physical-chemical parameters from aerobic ΔSterne-1(pPA102)CR4 fermentation. Symbols: open circles, DO₂; solid line, pH; open squares, agitation; solid squares, percent oxygen. The tryptone addition during the fermentation is indicated by the arrow above the percent oxygen data at 200 min. The temperature range was 36.7 to 37.4°C. Pressure was constant at 2.0 lb/in², and the sparge rate was 1 vol/vol/min. The sharp positive spike in the DO₂ values at 350 min was due to the addition of antifoam KFO673.

**FIG. 3.** PA production versus cell density. Symbols: solid circles, growth data 7 July fermentation; open circles, growth data 4 August fermentation; solid triangles, PA yield data 7 July fermentation; open triangles, PA yield data 4 August fermentation. Equivalent volumes were sampled from the 20-liter fermentor for each EFT. The OD₆₀₀ was determined for each EFT, and the samples were sterile filtered. The filtrates were desalted and concentrated to equivalent final volumes before a 1:1 dilution with twofold-concentrated SDS solubilization buffer and analysis by SDS-PAGE. Coomassie blue-stained gels were digitally scanned as described in Materials and Methods, and amounts of *Mₚ* 83,000 PA were determined for each EFT.
carbonic anhydrase, 31,000; trypsin inhibitor, 21,500; and lysozyme, 14,400.

were used: phosphorylase b, peroxidase. Bio-Rad low-molecular-weight standards with the following 1-naphthol after incubation with goat-anti mouse antibody linked to horseradish peroxidase. Bio-Rad low-molecular-weight standards with the following 

6 h; 8, immunoblot of EFT 6 h developed with a pool of four monoclonal antibodies. Equivalent sample volumes were taken for each EFT. Samples were sterile filtered, and the filtrate was desalted, lyophilized, and resuspended in buffer to the same final volume before a 1:1 dilution with twofold-concentrated SDS solubilization buffer and analysis by SDS-PAGE. Equal volumes of each buffer to the same final volume before a 1:1 dilution with twofold-concentrated SDS solubilization buffer and analysis by SDS-PAGE. Equal volumes of each buffer to the same final volume before a 1:1 dilution with twofold-concentrated SDS solubilization buffer and analysis by SDS-PAGE. Equal volumes of each 

aerobic fermentation of D Sterne-1(pPA102)CR4. Lanes: 1, molecular weight standards; 2, EFT 1 h; 3, EFT 2 h; 4, EFT 3 h; 5, EFT 4 h; 6, EFT 5 h; 7, EFT 6 h; 8, immunoblot of EFT 6 h developed with a pool of four monoclonal antibodies. Equivalent sample volumes were taken for each EFT. Samples were sterile filtered, and the filtrate was desalted, lyophilized, and resuspended 

Purification. The crude fermentation supernatant was sterilized by filtration and then concentrated and diafiltered into ammonium acetate buffer with ultrafiltration spiral-wound cartridges with a molecular mass cutoff of 30 kDa. The diafiltration step was introduced primarily for buffer exchange but was also a critical purification step, as shown by the decrease in the UV absorbance seen in Fig. 5. The decreased UV absorbance reflected the loss of protein, DNA, and RNA. A total diafiltration volume corresponding to 10 times the volume of the protein concentrate was required for the maximum 70- to 80-fold reduction in the UV absorbance seen in Fig. 5. SDS-PAGE analysis of the permeate, shown in the inset in Fig. 5, revealed an enrichment in proteins with 

yield of the product was 90 to 95%. Analysis of the $A_{260}/A_{280}$ ratio for the diafiltered sample containing the product revealed a value of 1.93, which indicates protein that was still contaminated with 69% (by weight) of nucleic acids (8, 26). The diafiltered protein and nucleic acid mixture was subjected to anion-exchange chromatography with a quaternized-amine resin to remove residual nucleic acid and peptide contaminants. To remove these contaminants without losing the product, we increased the conductivity of the protein mixture to 11 mS/cm with KCl before applying it to the resin. Under these conditions, the PA was not bound by the resin but nucleic acids and other protein contaminants were adsorbed. The $A_{260}/A_{280}$ ratio of the eluted protein was 0.68, which corresponded to a residual nucleic acid content of 1% (8, 26). The total protein recovery was between 50 and 55% of the amount loaded, while the $M_r$ 83,000 PA recovery was 85 to 90%. Once the nucleic acid contamination was removed, the partially purified protein was diafiltered into ammonium acetate

FIG. 4. SDS-PAGE results reflecting the time course of PA production from aerobic fermentation of D Sterne-1(pPA102)CR4. Lanes: 1, molecular weight standards; 2, PA after Macro-Prep 50 Q chromatography; 3, PA after HQ chromatography; 4, PA after QE chromatography; 5, PA after ether HIC chromatography. PA samples from each purification step were desalted, concentrated, and solubilized in SDS buffer. The total protein loaded in lanes 2 to 5 was 2 µg. Molecular weight standards were as in Fig. 4.

FIG. 5. UV-spectrophotometric and SDS-PAGE analysis of the diafiltration permeate. Symbols: solid circles, $A_{260}$; open circles, $A_{280}$. Permeate from the Amicon 30-kDa-cutoff spiral-wound cartridge during the first diafiltration step was collected, diluted with the diafiltration buffer, and analyzed by UV spectrophotometry at 280 and 260 nm. The spectrophotometer was blanked against the diafiltration buffer. Values at 350 and 320 nm were measured to confirm that sample turbidity was negligible. The inset shows a Coomassie blue-stained SDS-PAGE gel of a 10-fold concentrate of the diafiltration permeate collected at the outset of the diafiltration step.

FIG. 6. SDS-PAGE analysis of PA purity after chromatographic purification steps. Lanes: 1, molecular weight standards; 2, PA after Macro-Prep 50 Q chromatography; 3, PA after HQ chromatography; 4, PA after QE chromatography; 5, PA after ether HIC chromatography. PA samples from each purification step were desalted, concentrated, and solubilized in SDS buffer. The total protein loaded in lanes 2 to 5 was 2 µg. Molecular weight standards were as in Fig. 4.
buffer (pH 10) and concentrated approximately fourfold with a spiral-wound 30-kDa cutoff ultrafiltration membrane. The starting material for the HQ anion-exchange quaternized polyethyleneimine HPLC column is shown in Fig. 6, lane 2. The substantial purification achieved before HPLC chromatographic steps can be seen by comparing Fig. 6, lane 2, with Fig. 4, lane 7.

The partially purified material was applied to an HPLC HQ quaternized-amine ion-exchange column and eluted with a 10-column-volume linear ammonium acetate gradient. The \( M_r \) 83,000 PA was resolved from the majority of the contaminating polypeptides that failed to adsorb under these conditions and from two other peaks, which eluted before and after PA. The approximately 50 to 60% pure PA (Fig. 6, lane 3) was then diluted to 15 mS/cm with MQ water and applied to a QE quaternized-polyethyleneimine perfusion anion-exchange resin with a lower charge density and different selectivity than the HQ resin. At this pH, a single asymmetric peak was eluted with a 10-column-volume linear ammonium acetate gradient. The \( M_r \) 83,000 PA eluted at the front end, while the major impurities eluted later in the gradient as a pronounced shoulder. This fractionation of the main peak resulted in recovery of 35 to 40% of the initial \( M_r \) 83,000 PA present in the fermentor and a product that was typically 88 to 93% pure (lane 4).

Although the QE-purified \( M_r \) 83,000 PA fulfilled the initial goal of greater than 85% purity, we later determined that higher purity could be achieved by HIC, an orthogonally related chromatographic separation technique with separation based on hydrophobicity. The method was developed by using an ether-based HIC resin and a pH 10 buffer composed of ammonium sulfate and ammonium acetate. Under these initial buffer conditions, the \( M_r \) 83,000 PA bound to the column at 22 to 23°C while the remaining impurities eluted with an isocratic wash. The \( M_r \) 83,000 PA (Fig. 6, lane 5) was eluted with a linear gradient, although step gradient elution was also possible. The recovery of \( M_r \) 83,000 PA from this step was 90%, and the final purity was 95 to 98%. Although there was a previous report of an HIC method for PA, it proved difficult to compare the techniques because the temperature range and recovery for the previously reported method were not defined (38). In our hands, the ether HIC proved superior in final product purity and recovery to that of the previous report.

**Analysis of product purity.** The final product purity described above was determined by SDS-CE and reversed-phase chromatography. Figure 7A shows the SDS-CE analysis of QE-purified recombinant PA monitored at 215 nm. The main \( M_r \) 83,000 PA peak at 10.8 min is labeled 3, while the other major peak, labeled 1, at 3.65 min is the mellitic acid internal standard. The impurities can be seen as multiple single and double peaks collectively labeled 2 between 7.5 and 10.5 min. The peak labeled 4 at 11.99 min was investigated further and found to be identical to a contaminant with an \( M_r \) of 80,000 by SDS-PAGE. The reason for the increased estimated mass relative to the \( M_r \) 83,000 PA with SDS-CE was not clear, but the observed baseline resolution of SDS-CE made an assay of this 1 to 3% contaminant possible. Overall integration of the peak area of the \( M_r \) 83,000 PA and the sum of all the contaminants yielded a final PA purity of 90% for this lot. Analysis of the same PA after HIC purification (Fig. 7B) revealed a final purity of 98% with trace contaminants between 8 and 10.5 min at the threshold of detection. The only significant resolved contaminant peak was seen as a shoulder of the PA peak at 10.6 min, which was believed to be a minor related impurity that was also visualized as a slightly faster-migrating minor band by SDS-PAGE and immunoblotting (data not shown).

**FIG. 7.** Determination of PA purity by SDS capillary electrophoresis. PA samples were analyzed after QE (A) or ether HIC (B) chromatography steps. The samples were desalted and concentrated to 3 to 4 mg/ml before being solubilized with SDS as described in Materials and Methods. The samples were applied by electrokinetic injection at ~5 kV for 5 to 10 s. Separations were performed at ~10 kV for 15 min, and the \( A_{215} \) was monitored.
Purified PA was also analyzed by reversed-phase chromatography. PA lost solubility in the presence of acidic reversed-phase ion-pairing agents, necessitating the development of an alkaline reversed-phase method. The reversed-phase separation of QE-purified PA with 0.05% (wt/vol) NaOH as the ion-pairing agent is shown in Fig. 7B. The small peak at the beginning of the chromatogram is due to added EDTA and was not included in the total integrated peak area. The series of three peaks collectively labeled 1 and the main peak, labeled 2, were collected, concentrated, and further analyzed by SDS-PAGE. The polypeptides were collected into sufficient HEPES (pH 7.3) to avoid prolonged exposure and breakdown of the peptide backbone under the alkaline conditions. The results of the SDS-PAGE analysis are shown as the inset in Fig. 8. After concentration, the pooled multiple peaks (peak 1) contained six major polypeptide contaminants (lane 1 of inset) while peak 2 contained the \( M_r 83,000 \) PA (lane 2 of inset). Although the resolution value of the \( M_r 83,000 \) PA from the nearest contaminant peak was greater than 2.1, some \( M_r 83,000 \) PA coeluted with peak 1. The reason for this remains unclear, but it is possible that this PA was partially denatured. Significantly, reapplication of \( M_r 83,000 \) PA in peak 2 to the reversed-phase column resulted in a single peak with a retention time identical to that of the initial peak 2, making it unlikely that the reversed-phase method caused the changes resulting in the earlier elution pattern. Reversed-phase analysis of the same lot of HIC-purified PA shown in Fig. 7B revealed the same 98% purity level found by SDS-CE (data not shown).

Several different lots of purified PA were subjected to this reversed-phase separation to determine the presence of related and unrelated impurities after the QE stage of the purification. Figure 9 shows an immunoblot of the pooled protein contaminants in peak 1 from three separate lots. The immunoblot was probed with a mixture of four monoclonal antibodies, which had been mapped to different domains of PA (21–23) and visualized with the ECL system. The immunoreactivity of the polypeptides in Fig. 9 confirmed that 7 to 9% of the total impurities remaining in the QE-purified PA that were separated by reversed-phase chromatography were related impurities. The percentage of related impurities and distribution remained constant in stability studies, suggesting that protease activity was removed from the \( M_r 83,000 \) PA during purification (data not shown).

The higher-molecular-weight immunoreactive species was \( M_r 83,000 \) PA, while the major \( M_r 80,000 \) protein contaminant seen in Fig. 8 (inset lane 1) was not immunoreactive. This suggested that the \( M_r 80,000 \) contaminant which was present at 1 to 3% in the QE-purified PA was an unrelated impurity. The \( M_r 80,000 \) protein was purified by reversed-phase chromatography and subjected to N-terminal sequencing. The N-terminal sequence was determined to be N-ETLKE...C, while the \( M_r 83,000 \) PA isolated in the same manner yielded an N-terminal sequence of N-EVKQEN...C, which corresponded exactly to the DNA-derived amino acid sequence of PA. The sequence of the \( M_r 80,000 \) impurity did not correspond to the plasmid-encoded neomycin resistance gene product (28) or to any known \( B. anthracis \) proteins. The identity of the unrelated impurity remains unknown, since a search of the current protein data banks revealed no significant homologies.

Native PAGE of purified recombinant PA revealed the presence of microheterogeneity in the final product in the form of three major isoforms that were visualized as separate bands (data not shown). The presence of these isoforms was described previously for PA purified from the attenuated \( B. anthracis \) Sterne strain (20). Comparing the recombinant PA with PA from the Sterne strain revealed the same isoforms in both, although the recombinant was enriched in the upper three of the five total isoforms (data not shown). To determine whether the isoforms were the result of N- or C-terminal proteolysis, the recombinant PA that had three isoforms was described previously for PA purified from the attenuated \( B. anthracis \) Sterne strain (20). Comparing the recombinant PA with PA from the Sterne strain revealed the same isoforms in both, although the recombinant was enriched in the upper three of the five total isoforms (data not shown). To determine whether the isoforms were the result of N- or C-terminal proteolysis, the recombinant PA that had three isoforms was subjected to both N- and C-terminal sequencing. The sequence data from the N terminus were N-EVKQEN...C, while the C terminus was N-.... FSSKKGYEIG-C. Data from both termini yielded single conclusive sequences that correlated exactly with the DNA-derived amino acid sequences.

The biological activity of the recombinant PA was monitored by the macrophage lysis assay (6) against PA purified by the original protocol from the Sterne strain as a control (19). The cytotoxicity assay was performed by titrating the amount of PA added to the \( J774 \)A.1 cells with 40 ng of LF per ml and measuring the cell viability. The titration curve generated with the control Sterne PA was comparable to that generated with multiple lots of the purified recombinant PA (data not shown).
The amount of Sterne or recombinant PA required to kill 50% of the cells was 8 to 9 and 3 to 5 ng/ml, respectively. Because the curves and 50% control values were comparable for PA from both sources, the biological activity was clearly not adversely affected by the production or purification methods described here.

**DISCUSSION**

The major rationale for the continued use of a *B. anthracis* expression system was the direct secretion and accumulation of the desired protein into culture medium in a relatively pure state. In addition, the secretion apparatus was the native secretion system for PA, ensuring that the secreted protein would have the signal sequence correctly removed and that the protein would be correctly folded. An additional rationale for selecting the *B. anthracis* strain was the observed low protease activity in culture supernatants. The greater than 95% inhibition of supernatant protease activity by EDTA and the low (less than 30%) inhibition by PMSF alone made it unlikely that *B. anthracis* excreted significant subtilisin or related alkaline protease activity under the conditions used here. In addition, the lack of additional inhibition when PMSF, benzamidine, or 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) was added in addition to EDTA suggested the presence of fewer proteases under the growth conditions and medium used here than observed for other bacilli such as *B. subtilis* (1, 34). The inhibition of EDTA was consistent with a neutral or metalloprotease(s) (16, 27), although the presence of a calcium-dependent, serine-type protease (2, 34), as described for *B. subtilis*, could not be ruled out. The plausibility of omitting PMSF from the entire process was confirmed when no loss of product, increase in related impurities, or decrease in stability upon storage at 4°C was observed when only EDTA was added. The lack of a PMSF requirement was a key factor in the selection of *B. anthracis* as a host over *B. subtilis*, since the addition of this toxic inhibitor was essential for stable PA when it was isolated from *B. subtilis* strains. This included the recombinant strain *B. subtilis* WB600, which had six unique protease genes deleted but still required PMSF addition to inhibit its remaining activity (50).

Fermentation of the asporogenic variant ΔSterne-1(pPA102) CR4 under the conditions described here resulted in no detectable spore production and had the secondary effect of greatly reducing the amounts of surface array protein normally released into the supernatant under the conditions used here (5). The reduction in the amount of the surface array protein was not investigated further but was attributed to pleiotropic effects of the spontaneous mutation selected on Congo Red or to a second, uncharacterized mutation.

Comparison of growth in complete medium, reduced yeast extract, or reduced tryptone revealed no change in cell density or growth rate, suggesting that the observed decrease in oxygen use observed at approximately 10 to 12 OD₆₀₀ units in complete medium was not attributable to substrate limitation. Attempts at medium supplementation at this point did not increase overall cell densities or eliminate the drop in oxygen consumption. These observations were most consistent with the accumulation of a toxic metabolic by-product rather than the shortage of a critical nutrient as the cause of the observed cessation of growth. Because the aerobic fermentation of amino acids and peptides increases culture pH due to released ammonium, the ammonium levels (47) generated during fermentation were determined (data not shown). The final concentration of ammonium found during the fermentations and a twofold-higher concentration were tested as potential growth inhibitors by adding ammonium sulfate to *B. anthracis* cultures. Neither concentration had any effect on growth, suggesting that the accumulation of ammonium was not the reason for the cessation of growth under the fermentation conditions described here.

Although maintaining the high DO₂ set point of 75% optimized the growth rate, it may also have led to the observed limitation in cell density by facilitating the rapid accumulation of metabolic by-products. However, the susceptibility of PA to proteolytic degradation even in the presence of tryptone necessitated the development of a rapid fermentation process. The rationale for optimizing growth rates can be seen from the Mᵣ 83,000 PA yield data. Clearly, the yield of Mᵣ 83,000 PA reached a peak during the deceleration (early stationary) phase and then declined rapidly. This decline in the Mᵣ 83,000 PA level directly reduced yield and complicated the purification of Mᵣ 83,000 PA from the increasingly complex mixture of proteolytic degradation products.

Our results demonstrated that tryptone was essential for reducing the measured protease activity and maximizing product recovery at the end of the fermentation. SDS-CE analysis of filtered, nondialyzed tryptone revealed the presence of multiple species, with the majority of the material having a mass less than 15 kDa with minor contributions of higher-mass species. The addition of completely hydrolyzed protein in the form of Casamino Acids was ineffective in protecting PA from proteolytic degradation. These results suggest that it was the polypeptides in tryptone that were crucial to the observed effects on blocking of protease activity. It remains unclear whether the proteolysis is blocked by polypeptides interacting with the protease active site(s) or whether tryptone contributes to a reduction in the actual amounts of protease released into the medium by this organism.

The combination of diafiltration and the initial ion-exchange chromatography resulted in the removal of more than 99% of the nucleic acids and 70% of the carbohydrates. These steps also contributed significantly to the overall purification by eliminating up to 50% of the contaminating protein in the crude fermentation liquor while Mᵣ 83,000 PA recoveries were consistently around 90%. The subsequent ion-exchange purification yielded a product with 88 to 93% purity. The major losses in the whole process occurred during the second of these column purifications, with recoveries of 30 to 40% of the total initial product. These losses were incurred in part because of microheterogeneity in PA, which is readily observed in the form of multiple discrete bands or isoforms by native PAGE (19). The isoforms with higher mobility on native gels coeluted with lower-Mᵣ proteins, reducing the yield, while additional losses were incurred due to coelution of the Mᵣ 80,000 nonrelated impurity with the isoforms of lower mobility. The net result was a loss of 40 to 50% of the PA recovered from the HQ ion-exchange step and a product enriched in three of the initial isoforms.

The alkaline pH used during the purification was chosen for three reasons: (i) to avoid the pH optimum for the remaining contaminating protease(s), (ii) to maintain a sufficient difference from the pl of PA to minimize charge differences between isoforms, and (iii) to minimize protein-protein interactions between intact PA and proteolytic degradation products. The alkaline buffers were instituted with caution since biological activity was a criterion for final product and since exposure of PA to alkaline pH values greater than 8.9 to 9.0 was not previously reported. Comparing the biological activity of samples of PA purified under the conditions described here with PA purified under more physiological pH conditions revealed equivalent activities in the macrophage lysis assay. The data
suggest that the exposure to alkaline conditions did not affect the native folding state of PA, or did so in a manner that was not apparent in the cell lysis assay. We also compared the isof orm content to that of PA purified under physiological pH conditions, since prolonged exposure of proteins to alkaline pH can drive the nonenzymatic deamination of asparagine residues to aspartate and isoaspartate residues (33). No shift in the isof orm content or in the relative proportions of the iso forms was observed after exposure to buffers used here.

We also investigated the HIC method as a substitute for QE chromatography. The M, 83,000 PA was purified from the multiple small contaminants with excellent yield, but the product was simultaneously enriched with M, 37,000 and M, 47,000 proteolytic fragments of PA that did not dissociate under non denaturing conditions (32). The copurification of the proteolytically cut species of PA indicated that the HIC method was most useful as a final step.

Clearly, the investigation reported here was undertaken to improve on the undesirable aspects of the current vaccine production system. The production process described here successfully met a number of those goals, including production from an avirulent, asporogenic, nontoxic strain; fermentation in the absence of added antibiotic; and minimization of product proteolysis without the addition of the toxic protease inhibitor PMSF. In addition, we designed a 4-day purification process which is robust and flexible enough to overcome potential changes in the amount and ratio of impurities in the starting material yet produces the desired product in adequate yield and desired purity at the desired scale. The purities given starting material yet produces the desired product in adequate potential changes in the amount and ratio of impurities in the process which is robust and flexible enough to overcome po-

product proteolysis without the addition of the toxic protease

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