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Production, recovery and immunogenicity of the protective antigen from a recombinant strain of *Bacillus anthracis*

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The protective antigen (PA) is one of the three components of the anthrax toxin. It is a secreted nontoxic protein with a molecular weight of 83 kDa and is the major component of the currently licensed human vaccine for anthrax. Due to limitations found in the existing vaccine formulation, it has been proposed that genetically modified PA may be more effective as a vaccine. The expression and the stability of two recombinant PA (rPA) variants, PA-SNKE-ΔFF-E308D and PA-N657A, were studied. These proteins were expressed in the nonsporogenic avirulent strain BH445. Initial results indicated that PA-SNKE-ΔFF-E308D, which lacks two proteolysis-sensitive sites, is more stable than PA-N657A. Process development was conducted to establish an efficient production and purification process for PA-SNKE-ΔFF-E308D. pH, media composition, growth strategy and protease inhibitors composition were analyzed. The production process chosen was based on batch growth of *B. anthracis* using tryptone and yeast extract as the only source of carbon, pH control at 7.5, and antifoam 289. Optimal harvest time was 14–18 h after inoculation, and EDTA (5 mM) was added upon harvest for proteolysis control. Recovery of the rPA was performed by expanded-bed adsorption (EBA) on a hydrophobic interaction chromatography (HIC) resin, eliminating the need for centrifugation, microfiltration and diafiltration. The EBA step was followed by ion exchange and gel filtration. rPA yields before and after purification were 130 and 90 mg/l, respectively. The purified rPA, without further treatment, treated with small amounts of formalin or adsorbed on alum, induced, high levels of IgG anti-PA with neutralization activities.
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Introduction

Anthrax, a potentially fatal disease, is caused by *Bacillus anthracis*. The virulence of this pathogen is mediated by a capsule of a poly-D-γ-glutamic acid and an exotoxin composed of three proteins [15,17,18]. The three protein components are the protective antigen (PA, 83 kDa), lethal factor (LF, 90.2 kDa) and edema factor (EF, 88.8 kDa) These proteins, nontoxic by themselves, form lethal toxins when combined with an activated PA [17]. The genes coding for these three protein components and the capsule are found in the endogenous plasmids pXO1 and pXO2, respectively [33].

There is only indirect evidence, albeit extensive, identifying the components of vaccine-induced immunity to anthrax and there is evidence that anti-PA neutralizing antibody titers can be a reliable surrogate marker for protective immunity [27]. The PA seems to be an essential component of all vaccines for anthrax [8,20,34]: both mono and polyclonal antibodies to PA neutralize the anthrax toxin and confer immunity to *B. anthracis* in animal models. The US licensed vaccine for anthrax "Anthrax Vaccine Adsorbed" (AVA) is produced from the formalin-treated culture supernatant of *B. anthracis* Sterne strain, V770-NP1-R ($pXO1^+$, $pXO2^-$), adsorbed onto aluminum hydroxide [26]. Although AVA has been shown to be effective against cutaneous infection in animals and humans and against inhalation anthrax by rhesus monkeys [13], it has several limitations: (1) AVA elicits a relatively high degree of local and systemic adverse reactions probably mediated by variable amounts of undefined bacterial products, making standardization difficult; (2) the immunization schedule requires administration of six doses within an 18-month period, followed by annual boosters for those at risk; and (3) there is no defined vaccine-induced protective level of serum PA to evaluate new lots of vaccines.

Development of a well-characterized, standardized, effective and safe vaccine that would require fewer doses to confer immunity to both inhalational and cutaneous anthrax is needed [10,34]. It has been suggested that a vaccine composed of modified purified recombinant PA (rPA) would be effective, safer, allow precise standardization and probably would require fewer injections [31]. Such a PA can be designed to be biologically inactive, more stable and still maintain high immunogenicity.

We report the development of a production and purification process for rPA from the nonsporogenic avirulent *B. anthracis* BH445 ($pXO1^-$, $pXO2^-$) strain. Following an 18-h fermentation and three purification steps, large quantities of PA suitable for vaccine production were obtained. The purified rPA was tested in mice for its ability to elicit neutralizing antibodies.

Materials and methods

Strains and plasmids

The nonsporogenic, protease deficient, avirulent strain *B. anthracis* BH445 ($pXO1^-$, $pXO2^-$, cm^r) was used [18]. The *Bacillus–E. coli* shuttle vector pYS5 (amp^r, kan^r) [30] was used to clone two

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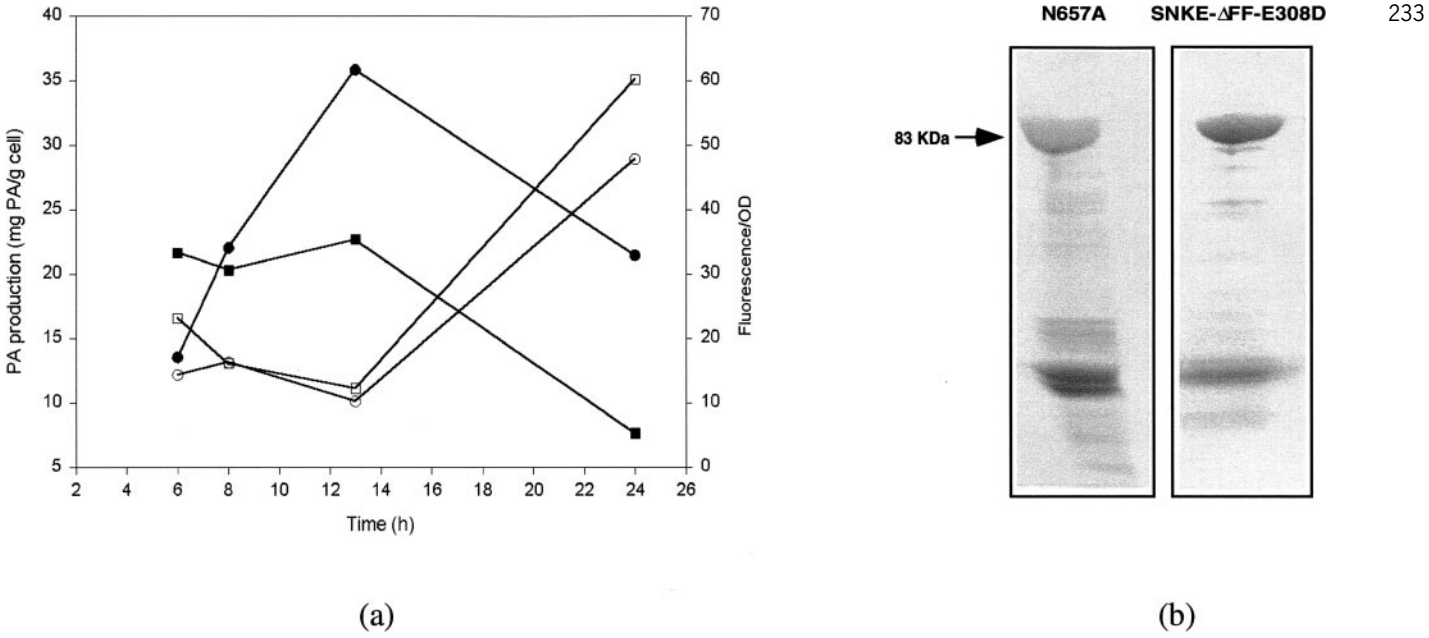

Figure 1 Production and proteolytic activity of PA-SNKE-ΔFF-E308D and PA-N657A. (a) rPA production (mg/g cells) (●) SNKE, (■) N657A; proteolytic activity (○) SNKE, (□) N657A; (b) SDS-PAGE analysis of partially purified PA-N657A and PA-SNKE-ΔFF-E308D.

recombinant forms of the PA: N657A and SNKE-ΔFF-E308D [32]. In the N657A mutant, the receptor-binding domain of PA was altered by substitution of Asp with Ala at position 657 (domain 4). In the SNKE-ΔFF-E308D mutant, two regions were altered, the furin site ($RKKR^{167}$ to $SNKE^{167}$) and the chymotrypsin site (two Phe at positions 313–314 were deleted and Glu at position 308 was substituted with Asp). Both rPA constructs contain the DNA sequence encoding the signal peptide of rPA.

Culture and expression conditions

Modified FA medium [24] containing (per liter) 35 g tryptone (Difco Laboratories, Detroit, MI), 5 g yeast extract (Difco Laboratories), and 100 ml of 10× salt solution was used in all experiments. The 10× salt solution consisted of (grams per liter) 60 $Na_2HPO_4{\cdot}7H2O$, 10 KH_2PO_4, 55 NaCl, 0.4 L-tryptophan, 0.4 L-methionine, 0.05 thiamine, and 0.25 uracil. It was filter-sterilized and added to the fermentor after it had cooled. The pH of the medium was adjusted to 7.5; 100 μg/ml kanamycin and 20 μg/ml chloramphenicol were added. Fermentation experiments were performed by inoculating a 12- to 14-h-old starter culture grown from a frozen stock. The medium in the fermentor was supplemented with 0.2 ml/l of antifoam 289 (Sigma, St. Louis, MO). Three- to ten-liter fermentations were carried out using a B. Braun Biostat MD DCU (Melsungen, Germany), controlling dissolved oxygen (DO) at 30% saturation, temperature at 37°C, and pH at 7.5 with HCl and NH4OH. At harvest time, 5 mM EDTA and 10 μg/ml PMSF (phenylmethyl sulfonyl fluoride) were added to the culture. Shake flask experiments (100 ml) utilizing modified FA medium were supplemented with glucose, lactose, glycerol or casitone at a concentration of 10 g/l.

Table 1 Effect of various carbon sources on rPA production

Medium	rPA production	
	mg rPA/g cell	mg rPA/l culture
Glycerol+basic medium	23.7	117.3
Glucose+basic medium	25.3	113.3
Lactose+basic medium	33.9	116.0
Casitone+basic medium	28.3	135.1

Analytical methods

Optical density (OD) was measured at 600 nm. Protease analysis was carried out on supernatant samples collected during growth and stored frozen at −20°C. EDTA was added to supernatant samples used for SDS-PAGE and radial immunodiffusion to a final concentration of 10 mM.

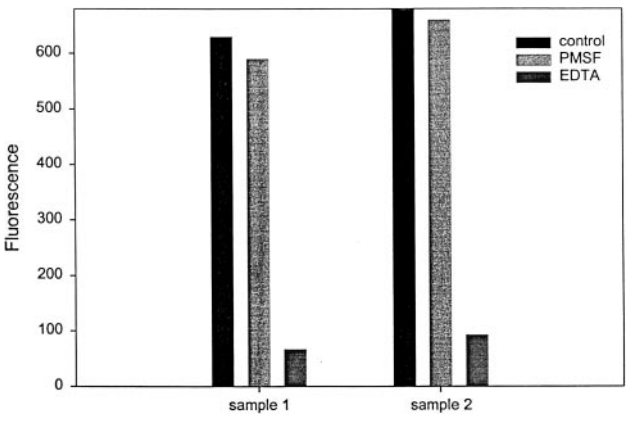

Figure 2 Effect of EDTA and PMSF on proteolytic activity. Supernatants from two different cultures taken after 24 h of growth were analyzed without inhibitors (control), with 1 μg/μL PMSF and with 15 mM EDTA. Fluorescence is proportional to proteolytic activity.

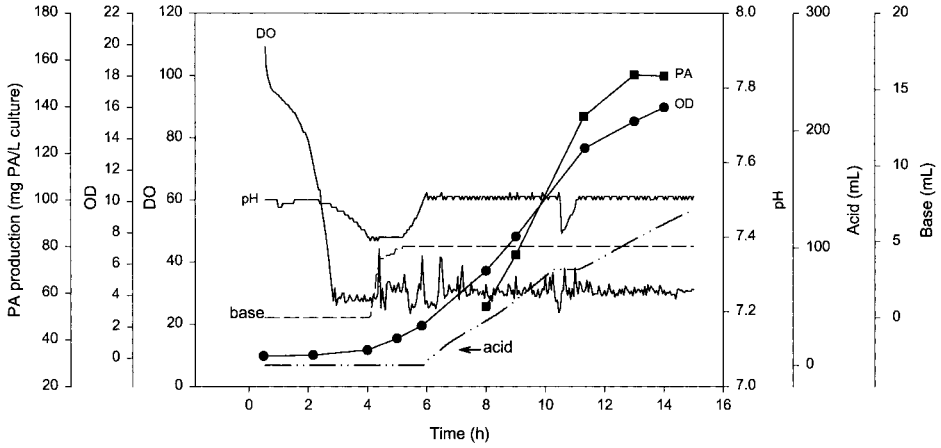

Figure 3 Fermentation process for the production of PA-SNKE-ΔFF-E308D from *B. anthracis* BH445. (■) rPA (mg/l), (●) OD 600 nm, (– – – –) base addition (ml), (– ·· – ·· –) acid addition (ml). Dissolved oxygen and pH are depicted as solid lines.

Extracellular protease activity was detected using the EnzChek green fluorescence assay kit (Molecular Probes, Eugene, OR). Fluorescence was measured with a LS50B luminescence spectrophotometer (Perkin-Elmer, Boston, MA). This assay was conducted at pH 7.5 or 6.0 depending on the experiment. Proteolytic activity is reported as fluorescence change per unit sample.

Protein was determined using BCA assay (Pierce, Rockford, IL). PA expression was quantified by SDS-PAGE (Invitrogen/Novex, Carlsbad, CA) gel analysis and by the Mancini immunodiffusion assay [21] using agarose plates containing polyclonal PA antibody. Pure PA was used as the standard, both polycolonal PA antibodies and pure PA were supplied by S. Leppla (NIH, NIDCR, Bethesda, MD, USA).

Immunological methods

Immunogens used: (1) purified rPA; (2) formalin-treated rPA: rPA, at concentrations of 0.3, 3.0 and 7.12 mg/ml, was treated with 0.035% formalin [23,25]. The reaction mixtures were dialyzed extensively against phosphate-buffered saline, pH 7.4 and sterile-filtered through a 0.2-μm pore-size membrane; (3) rPA alum adsorbed: rPA at a concentration of 25 μg/ml was adsorbed onto 0.75 mg/ml aluminum hydroxide (Alhydrogel, SuperFos, Copenhagen, Denmark); (4) AVA US licensed vaccine: Lot # FAV 057.

Immune response analyses: In a representative experiment designed to characterize the immunogenicity of rPA, 5- to 6-week-old female general-purpose mice were injected subcutaneously three times two weeks apart [3] with 2.5 μg of the various rPA preparations (1/10 to 1/5 of the estimated human dose) in 0.1 ml of saline, and with 0.1 ml (1/5 of recommended dose) of the AVA. The mice were exsanguinated 7 days after the last injection and their IgG anti-PA measured by ELISA using native PA as the antigen and calculated in reference to a monoclonal anti-PA. The results are expressed as micrograms Ab per milliliter. Select sera were also assayed for their neutralization titer [19].

Purification

Packed-bed hydrophobic interaction chromatography:

The cell suspension containing 5 mM EDTA was centrifuged and

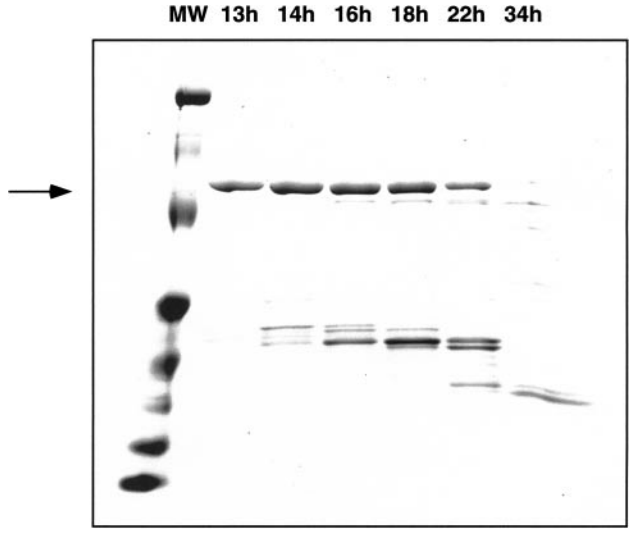

Figure 4 SDS-PAGE analysis of culture supernatants obtained throughout the fermentation. Samples were taken at 13, 14, 16, 18, 22 and 34 h of growth. Arrow indicates the location of rPA(83 kDa) in the gel.

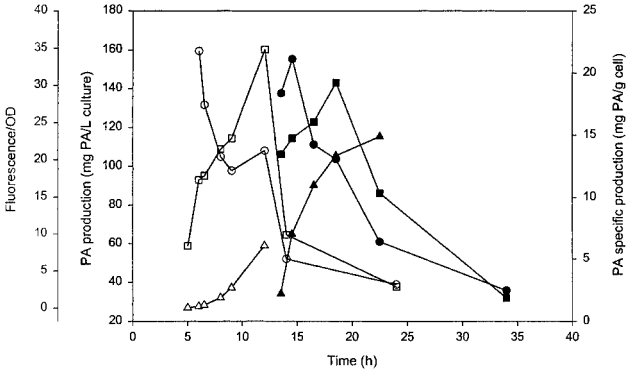

Figure 5 rPA production and proteolytic activity of *B. anthracis* BH445 [pSY5:SNKE-ΔFF-E308D] in fed-batch cultures supplied with tryptone/yeast extract or glucose. (●) Specific rPA production in tryptone/yeast extract (mg/g cells); (■) volumetric rPA production in tryptone/yeast extract (mg/l); (▲) proteolytic activity in tryptone/yeast extract; (○) specific rPA production in glucose (mg/g cells); (□) volumetric rPA production in glucose (mg/l); (△) proteolytic activity in glucose.

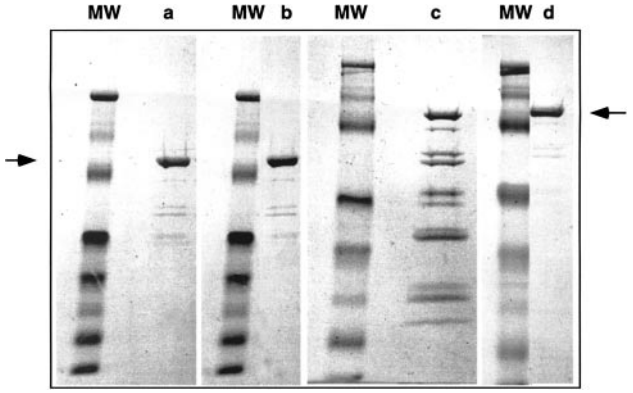

Figure 6 SDS-PAGE analysis of purified rPA fractions. (a) rPA purified by packed-bed chromatography; (b) rPA after hydrophobic interaction chromatography and gel filtration; (c) rPA fraction shown in lane (b) after 3 months; (d) rPA after expanded-bed hydrophobic interaction chromatography, anion exchange, and gel filtration. MW indicates molecular weight markers. Arrows indicate the location of rPA (83 kDa) in the gel.

the supernatant was passed through a 0.2-μm hollow fiber filter (AGT, Needham, MA). The filtered broth was then concentrated 20× using a 10K membrane in a Pellicon-2 (Millipore, Bedford, MA). Two hundred grams of $(NH_4)_2SO_4$ per liter (1.5 M) was added to the concentrated supernatant. The small amount of precipitate produced after addition of $(NH_4)_2SO_4$ was eliminated with centrifugation and filtration. Phenyl Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) was equilibrated with buffer containing 1.5 M $(NH_4)_2SO_4$/10 mM HEPES pH=7.0/5 mM EDTA (equilibration buffer) at a flow rate of 15 cm/h. After sample loading, the column was washed with 10 column volumes (CV) of equilibration buffer and rPA was eluted with a 30-CV linear gradient from 1.5 M to 0 M $(NH_4)_2SO_4$ in 10 mM HEPES pH=7.0/5 mM EDTA. Fractions were analyzed by SDS-PAGE and the rPA-containing samples were pooled for further purification.

Expanded-bed hydrophobic interaction chromatography: The cell suspension containing 5 mM EDTA was diluted 1:1 with buffer containing 3.0 M $(NH_4)_2SO_4$/20 mM HEPES pH=7.0/5 mM EDTA and 0.005% pluronic F-68 (Life Technologies, Gaithersburg, MD). STREAMLINE™ Phenyl, (Amersham Pharmacia Biotech) was expanded in a streamline column in equilibration buffer. The diluted cell suspension was loaded upward at 300 cm/h. The column was washed in expanded mode [2] with 10 CV of equilibration buffer containing 0.005% pluronic F-68. Elution was performed in packed-bed mode with 8 CV of elution buffer at 100 cm/h. The eluent was analyzed by SDS-PAGE and radial immunodifussion.

Anion exchange chromatography: Fractions from HIC were dialyzed against 20 mM Tris pH=8.9/5 mM EDTA and loaded on a Q Sepharose Fast Flow (Amersham Pharmacia Biotech) column equilibrated with 20 mM Tris pH=8.9/5 mM EDTA at 15 cm/h. The protein was eluted using a 20-CV linear gradient from 0 to 0.5 M NaCl in the same buffer. rPA-containing fractions were concentrated and dialyzed against PBS.

Gel filtration: The pooled rPA was further purified using a Superdex 75 column (Amersham Pharmacia Biotech) in PBS/5 mM EDTA pH 7.4 at 12 cm/h.

Results and discussion

Expression of two rPAs: PA-N657A and PA-SNKE-ΔFF-E308D

The expression of two recombinant versions of PA and the extracellular proteolytic activity of the culture were analyzed (Figure 1). Production of PA-SNKE-ΔFF-E308D, the protein lacking the furin and chymotrypsin cleavage sites, was nearly 60% higher than that of PA-N657A, the protein containing a mutation in the receptor-binding domain (Figure 1a). The extracellular proteolytic activity (fluorescence/OD) of both cultures was similar. SDS-PAGE analysis of partially purified rPA recovered from these cultures shows a higher concentration of smaller fragments in the sample from PA-N657A compared to the sample from PA-SNKE-ΔFF-E308D (Figure 1b). Western blot analysis with polyclonal PA antibody confirmed that the smaller fragments were reactive against rPA (data not shown). As indicated in Figure 1a, the proteolytic activity was similar in both strains. Therefore, it was apparent that PA-SNKE-ΔFF-E308D is a better candidate, due to its stability, and it was selected for further studies.

pH effect

Based on previous information [6,24], initial production studies with PA-SNKE-ΔFF-E308D were done by controlling pH with NH_4OH only, which resulted in a pH of 8.7 at the end of the fermentation. When pH was controlled at 7.4 during the entire fermentation, the rPA production was 30 mg/g cell and the

Table 2 Comparison of packed-bed and expanded-bed adsorption as capturing processes for rPA

Packed-bed	Expanded-bed adsorption
1. Total processing time: 15.5 h	1. Total processing time: 8 h
(a) downstream processing: 6 h (4 unit operations)	(a) downstream processing: 1 h (1 unit operation)
(b) loading: 2 h	(b) loading: 4 h
(c) column wash: 3.5 h	(c) column wash: 1.5 h
(d) elution: 4 h	(d) elution: 1.5 h
2. 400 g $(NH_4)_2SO_4$ needed	2. 8000 g $(NH_4)_2SO_4$ needed
3. 100 ml resin needed	3. 300 ml resin needed
4. Load/wash steps require little attention	4. Load/wash steps cannot be left unattended
5. 82% recovery	5. 70% recovery

Table 3 IgG anti-PA (ELISA) and anthrax toxin neutralization titers of mice ($n=10$) following three subcutaneous injections of the rPA and its derivatives[a]

Immunogen	Dose (μg)	μg IgG of selected mice	GM[b] of group of 10 mice	Toxin neutralization titer
rPA	2.5	130.6	24.9	4000
		11.2		200
		21.3		1000
rPA/AL(OH)[c]	2.5	725.3	312	8000
		770.5		7000
		513.5		4000
rPA/formalin 0.3[c]	2.5	68.3	182	1500
		179.2		2000
rPA/formalin 3.0[c]	2.5	77.7	220	1250
		214.4		5000
rPA/formalin 7.12[c]	2.5	262.5	266	3000
		415		5000
AVA[d]	0.1 ml	339.2	565	3000
Saline	0.1 ml	<0.05	<0.05	20

[a]Five- to six-week-old female general-purpose mice were injected subcutaneously with 0.1 ml of the above immunogens three times two weeks apart. The mice were bled 7 days after the third injection and select sera were assayed for IgG anti-PA by ELISA, using PA as the antigen, and by toxin neutralization.
[b]Geometric mean of anti PA antibody.
[c]PA at a concentration of 0.3, 3.0 and 7.12 mg per ml was treated with 0.035% formalin.
[d]US licensed Anthrax vaccine Lot FAV 0 57 was used without further treatment.

proteolytic activity per OD unit was 8, compared to values of 20 mg rPA/g cells and proteolytic activity per OD of 30 when the pH was controlled only by NH_4OH. When the process was performed at a lower pH, both rPA production and protease activity were lower. At pH 6.1 production declined nearly six times and protease activity two times compared to what was found at pH 7.4. Possibly, intracellular expression is lower or secretion is inhibited at low pH. From the above information, it is obvious that pH significantly affects the proteolytic activity and the rPA expression. Controlling pH throughout the fermentation process resulted in a 30% increase in rPA yield, compared to previously reported strategies.

Effect of various carbon sources and protease inhibitors

Attempts to increase PA expression by supplementing the basic growth medium with different carbon sources is summarized in Table 1. Neither the volumetric production nor the production per gram cells was enhanced with the addition of various carbon sources. The effect of PMSF and EDTA on extracellular proteolysis was also examined. As shown in Figure 2, addition of EDTA (15 mM) significantly reduced proteolytic activity whereas the proteolytic activity of the PMSF-containing fraction (1 μg/μl) was similar to that of the control. Based on this information, EDTA was added at the end of the fermentation, before the protein was processed.

Growth and production conditions

Based on the parameters determined previously, a production process for the recombinant PA-SNKE-ΔFF-E308D from *B. anthracis* BH445 was established. The process is based on growth in a batch fermentation controlled at pH 7.5 with NH_4OH/HCl and at 30% dissolved oxygen saturation for a period of 18 h. Results of a typical fermentation are shown in Figure 3.

In general, the final OD_{600} values fluctuated between 16 and 20. During the first 5 h, growth was exponential and the pH was controlled by base addition. Later in the fermentation, pH was controlled by acid addition. Accumulation of rPA occurred mostly during the stationary phase and reached a final concentration of 160 mg/l. The results shown in Figure 4 indicate that rPA degraded if the fermentation was extended for more than 18 h, therefore, a harvest time between 14 and 18 h was selected.

Attempts to increase the rPA production by implementing a fed-batch growth strategy were conducted. The addition of 10× tryptone/yeast extract/salts or 50% glucose/10× salts resulted in a 50% increase in cell density but not an increase in protein production (Figure 5). The observation that rPA production was not improved by the implementation of a fed batch growth strategy or by the addition of various carbon sources such as casein, glucose, glycerol or lactose is an indication that perhaps a specific nutritional factor is missing. Moreover, the specific proteolytic activity was almost five times lower when glucose was added to the tryptone/yeast extract media (Figure 6). This was expected since glucose represses proteases in *Bacillus* [11,29].

Purification

The purification protocol developed for rPA consisted of hydrophobic interaction chromatography (Phenyl Sepharose) followed by anion exchange (Q Sepharose) and gel filtration (Superdex 75).

Replacing the initial capturing step with expanded-bed chromatography [2] can simplify and shorten the recovery process since it eliminates the clarification steps. Therefore, the use of expanded-bed adsorption (EBA) was investigated by substituting the traditional packed-bed resin (Phenyl Sepharose) with the expanded-bed hydrophobic resin STREAMLINE™ Phenyl. The static binding capacity for STREAMLINE™ Phenyl was approximately 15 mg protein/ml of resin, which is comparable to the capacity of Phenyl Sepharose. Optimal binding of rPA to STREAMLINE™ Phenyl occurred at 1.5 M $(NH4)2SO_4$.

Preliminary experiments performed with cell-containing broth in expanded mode resulted in the formation of aggregates and eventual collapse of the bed. It was possible to stabilize the expanded column only after the addition of a detergent, which probably altered some of the hydrophobic interactions but did not

prevent rPA from binding. Pluronic F-68 was chosen due to its nontoxicity in humans. The static binding capacities of STREAMLINE™ Phenyl were 15, 11 and 5 mg protein/ml resin with 0%, 0.005% and 0.01% pluronic F-68, respectively. Successful operation of the HIC EBA column occurred when using a load concentration of 15 g wet cells/l, 0.8 ml resin/g wet cells and 0.005% pluronic F-68 in the load as well as the wash buffer. Under these conditions, some signs of aggregation appeared at the end of the loading phase but cell debris was eliminated in the washing phase. A 70% recovery was obtained.

rPA purity after hydrophobic interaction chromatography was higher than 80%. Further purification was achieved by adding a gel filtration step (Figure 6, lane b). However, this material was not stable when stored at 4°C for 3 months (Figure 6, lane c). In contrast, pure and stable rPA was obtained after HIC EBA, followed by anion exchange and gel filtration (Figure 6, lane d). Similar results to the expanded-bed process were obtained when packed-bed hydrophobic interaction chromatography was followed by ion exchange and gel filtration (Figure 6, lane a).

Replacing the packed-bed capturing step with EBA proved to be more efficient since it eliminated the centrifugation and filtration steps, however, twenty times more $(NH_4)_2SO_4$ and three times more resin were required to process the same amount of culture (Table 2). Initial work with hydrophobic interaction chromatography using EBA to capture rPA resulted in bed collapse. This was avoided after the addition of a surfactant (pluronic F-68). These results suggest that the characteristics of the cell membrane were most likely the cause of cell aggregation. Since no polyglutamic acid capsule is present in the recombinant strain, the two hydrophobic membrane proteins forming the S-layer [5,7] may be responsible for associating with neighboring cell membranes and the resin. After evaluating the possible interactions affecting the system, it was found that successful operation of the expanded bed was possible by carefully adjusting the cell concentration of the load, increasing the adsorbent-to-cell ratio, and choosing the appropriate detergent type and concentration. The expanded-bed approach was more efficient in spite of the slightly lower yield (70% vs. 82%) and the higher amount of $(NH_4)_2SO_4$ and resin needed since it eliminated the need for centrifugation and filtration. To obtain stable and highly purified protein, anion exchange and gel filtration steps were added.

Immunological studies

The results of the immunological studies are summarized in Table 3. They showed that all vaccinated groups responded with significant IgG anti-PA levels. Recipients of the adsorbed rPA, formalin-treated rPA and AVA had higher antibodies levels than the recipients of the purified rPA (312, 182, 220, 266, 565 vs. 24.9, $P<0.0001$): there was no statistical difference between GM antibody levels of the groups injected with the formalin-treated rPA, the alum-adsorbed rPA and the AVA. Sera from the vaccinated mice had toxin neutralizing titers which correlated with their antibody levels as measured by ELISA (Kendall tau *B*, $r=0.647$; $P<0.0001$).

The vaccination was done at a dose and route relevant to immunization of humans (subcutaneously 1/10–2/10 of the estimated human dose). The purified rPA is expected to induce fewer adverse effects than the AVA, which is composed of the adsorbed and formalin-treated culture supernatant of a toxic, noncapsulated Stern strain of *B. anthracis*. In addition, the rPA, being a purified single component preparation, is easy to characterize and standardize.

Conclusions

Once the gene encoding PA (*pagA*) was cloned [35] and sequenced [36], several researchers reported on the expression of PA in hosts like *Bacillus subtilis* [1,14,22,30], *E. coli* [9,28,35], *Salmonella typhimurium* [4], viruses [12], and avirulant *B. anthracis* [6,16]. From these reports, the highest PA yield achieved has been in the order of 50 mg/l in *B. anthracis* [16]. In this work, a scalable fermentation and purification process suitable for vaccine development, which produced almost three times more product than reported earlier, is presented. This was accomplished by using a biologically inactive protease-resistant PA variant in a protease-deficient nonsporogenic avirulent strain of *B. anthracis*. Immunogenicity studies in mice indicated that the purified rPA induced high levels of IgG anti-PA with neutralization activities.

References

1 Baillie L, A Moir and R Manchee. 1998. The expression of the protective antigen of *Bacillus anthracis* in *Bacillus subtilis*. *J Appl Microbiol* 84: 741–746.
2 Chase HA. 1994. Purification of proteins by adsorption chromatography in expanded bed. *Trends Biotechnol* 12: 296–303.
3 Chu C, R Schneerson, JB Robbins, and SC Rastogi. 1983. Further studies on immunogenicity of *Haemophilus influenzae* type b and pneumococcal 6A polysaccharide–protein conjugates. *Infect Immun* 40: 245–256.
4 Coulson NM, M Fulop and RW Titball. 1994. *Bacillus anthracis* protective antigen expressed in *Salmonella typhimurium* SL3261 affords protection against anthrax spore challenge. *Vaccine* 12: 1395–1401.
5 Farchaus JW, WJ Ribot, MB Downs and JW Ezzell. 1995. Purification and characterization of the major surface array protein from the avirulent *Bacillus anthracis* Δ Sterne-1. *J Bacteriol* 177: 2481–2489.
6 Farchaus JW, WJ Ribot, S Jendrek and SF Little. 1998. Fermentation, purification, and characterization of protective antigen from a recombinant, avirulent strain of *Bacillus anthracis*. *Appl Environ Microbiol* 64: 982–991.
7 Fouet A, S Mesnage, E Tosi-Couture, P Gounon and M Mock. 1999. *Bacillus anthracis* surface: capsule and S-layer. *J Appl Microbiol* 87: 251–255.
8 Gladstone GP. 1946. Immunity to anthrax: protective antigen present in cell-free culture filtrates. *Br J Exp Pathol* 27: 394–418.
9 Gupta P, SM Waheed and R Bhatnagar. 1999. Expression and purification of the recombinant protective antigen of *Bacillus anthracis*. *Prot Expession Purif* 16: 369–376.
10 Hambleton P and PCB Turnbull. 1990. Anthrax vaccine development: a continuing story. *Adv Biotechnol Processes* 13: 105–122.
11 Hemila H, M Pokkinen and I Palva. 1992. Improving the production of *E. coli* β-lactamase in *Bacillus subtilis*: the effect of glucose, pH, and temperature on the production level. *J Biotechnol* 26: 245–256.
12 Iacono-Connors LC, CS Schmaljohn and JM Dalrymple. 1990. Expression of the *Bacillus anthracis* protective antigen gene by baculovirus and vaccinia virus recombinants. *Infect Immun* 58: 366–372.
13 Ivins BE, MLM Pitt, PF Fellows, JW Farchaus, GE Benner, DM Waag, SF Little, GW Anderson, PH Gibbs and AM Friedlander. 1998. Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques. *Vaccine* 16: 1141–1148.
14 Ivins BE and SL Welkos. 1986. Cloning and expression of the *Bacillus anthracis* protective antigen gene in *Bacillus subtilis*. *Infect Immun* 54: 537–542.

15 Keppie J, PW Harris-Smith and H Smith. 1963. The chemical basis of the virulence of *Bacillus anthracis*: IX. Its aggressins and their mode of action. *Br J Exp Pathol* 44: 446–453.
16 Leppla SH. 1988. Production and purification of anthrax toxin. *Methods Enzymol* 165: 103–116.
17 Leppla SH. 1991. The anthrax toxin complex. In: Alouf JE and JH Freer (Eds.), Sourcebook of Bacterial Toxins. Academic Press, San Diego, CA, pp. 277–302.
18 Leppla SH. 1995. Anthrax toxins. In: Moss J, B Iglewski, M Vaughan and A Tu (Eds.), Bacterial Toxins and Virulence Factors in Disease. Handbook of Natural Toxins. Marcel Dekker, New York, pp. 543–572.
19 Little SF, SH Leppla and AM Fridlander. 1990. Production and characterization of monoclonal antibodies against the lethal factor component of *Bacillus anthracis* lethal toxin. *Infect Immun* 58: 1606–1613.
20 Little SF, BE Ivins, PF Fellows and AM Friedlander. 1997. Passive protection by polyclonal antibodies against *Bacillus anthracis* infection in guinea pigs. *Infect Immun* 65: 171–5175.
21 Mancini G, AO Carbonara and JF Hermans. 1966. Immunological quantitation of antigens by single radial immunodiffusion. *Immunochemistry* 2: 235–354.
22 Miller J, BW McBride, RJ Manchee, P Moore and LW Baillie. 1998. Production and purification of recombinant protective antigen and protective efficacy against *Bacillus anthracis*. *Lett Appl Microbiol* 26: 56–60.
23 Nencioni L, G Volpini, S Peppoloni, M Bugnoli, T DeMagistris, I Marsili and R Rappuoli. 1990. Properties of the pertussis toxin mutant PT-9K/129G after formaldehyde treatment. *Infect Immun* 59: 625–630.
24 Park S and SH Leppla. 2000. Optimized production and purification of *Bacillus anthracis* lethal factor. *Prot Expression Purif* 18: 293–302.
25 Porro M, M Saletti, L Nencioni, L Tagliaferri, and I Marsili. 1980. Immunogenic correlation between cross-reacting material (CRM 197) produced by a mutant of corynebacterium diphtheria and diphtheria toxoid. *J Infect Dis* 142: 716–724.
26 Puziss M, LC Manning, LW Lynch, E Barclay, I Abelow and GG Wright. 1963. Large-scale production of protective antigen of *Bacillus anthracis* anaerobic cultures. *Appl Microbiol* 11: 330–334.
27 Reuveny S, MD White, YY Adar, Y Kafri, Z Altboum, Y Gozes, D Kobiler, A Shafferman and B Velan. 2001. Search for correlates of protective immunity conferred by anthrax vaccine. *Infect Immun* 69: 2888–2893.
28 Sharma M, PK Swain, AP Chopra, VK Chaudhary and Y Singh. 1996. Expression and purification of anthrax toxin protective antigen from *Escherichia coli*. *Prot Expression Purif* 7: 33–38.
29 Simonen M and I Palva. 1993. Protein secretion in *Bacillus* species. *Microbiol Rev* 57: 109–137.
30 Singh Y, VK Chaudhary and SH Leppla. 1989. A deleted variant of *Bacillus anthracis* protective antigen is non-toxic and blocks anthrax toxin action *in vivo*. *J Biol Chem* 264: 19103–19107.
31 Singh Y, BE Ivins and SH Leppla. 1998. Study of immunization against anthrax with the purified recombinant protective antigen of *Bacillus anthracis*. *Infect Immun* 66: 3447–3448.
32 Singh Y, KR Klimpel, N Arora, M Sharma and SH Leppla. 1994. The chymotrypsin-sensitive site, FFD315, in anthrax toxin protective antigen is required for translocation of lethal factor. *J Biol Chem* 269: 29039–29046.
33 Thorne CB. 1985. Genetics of *Bacillus anthracis*. In: Leive L, PF Bonventre, JA Morello, S Schlesinger, SD Silver and HC Wu (Eds.), Microbiology. American Society for Microbiology, Washington, DC, pp. 56–62.
34 Turnbull PCB. 1991. Anthrax vaccines: past, present, and future. *Vaccine* 9: 533–539.
35 Vodkin MH and SH Leppla. 1983. Cloning of the protective antigen gene of *Bacillus anthracis*. *Cell* 34: 693–697.
36 Welkos SL, JR Lowe, F Eden-McCutchan, M Vodkin, SH Leppla and JJ Schmidt. 1988. Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis*. *Gene* 69: 287–300.