Biochemical and Biophysical Research Communications **286,** 6–11 (2001)
doi:10.1006/bbrc.2001.5337, available online at http://www.idealibrary.com on IDEAL®

Rapid Purification of Recombinant Anthrax-Protective Antigen under Nondenaturing Conditions

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Received July 10, 2001

Anthrax-protective antigen is the central moiety of the anthrax toxin complex that mediates the entry of the other two toxin components, lethal factor and edema factor into the cells. It is also the main immunogen of the cell-free vaccine against anthrax. However, in addition to PA, the vaccine contains trace amounts of other culture-derived proteins that contribute to the side effects of the vaccine like pain, edema, erythrema, etc. Thus there is a need to develop high-resolution purification methods to purify PA to homogeneity. In this study we have presented a purification strategy for rapid purification of recombinant protective antigen under nondenaturing conditions, which ensures that not only biological activity but also the conformational integrity of immunological epitopes is well-preserved. The protein was purified to homogeneity in a two-step purification procedure that takes just 6 h for completion. Three milligrams of recombinant protective antigen obtained from 1-liter culture was comparable to *B. anthracis* protective antigen in terms of functional and biological activity. Moreover, the immunogenicity elicited by the purified protein in mice was also studied. The studies reported here are part of continuing research that aims to provide a safe and efficacious alternative to the current vaccine against anthrax. © 2001 Academic Press

Key Words: **anthrax protective antigen; rapid purification; ion exchange chromatography; hydrophobic interaction chromatography.**

Anthrax has played a lead role in the history of medical sciences. Dating back to the establishment of Koch postulates and the launch of the first bacterial vaccine, anthrax has come a long way to its current and controversial use as a biological warfare agent. Anthrax toxin (1), recognized as an important virulence factor of *B. anthracis*, refers to three-protein exotoxin, Protective antigen (PA, 83 kDa), Edema factor (EF, 89 kDa), and Lethal factor (LF, 90 kDa).

Protective antigen plays a central role during intoxication by anthrax (2), it being the receptor-binding moiety, facilitating the entry of activity moieties-EF and LF, into the cells (3–5). Binary combination of PA and LF, called anthrax lethal toxin, causes death in infected animals and macrophage-like cell line J774A.1 (6–8). The combination of PA and EF, called anthrax edema toxin, causes edema when injected intradermally in experimental animals (9). PA is being studied for its use as a protein delivery system, since it can also translocate hetrologous proteins.

Protective antigen, as the name suggests, is the main component of the cell-free vaccine against anthrax. In fact the UK and USA vaccines consists of alum-precipitated, cell-free filtrates of noncapsulating Sterne strain of cultures of *B. anthracis*, grown to maximize the PA content. Considerable efforts are being made for the production, purification, and thermostabilisation (10) of protective antigen.

The gene for protective antigen from *B. anthracis* has been cloned and sequenced (11, 12). *B. anthracis* has been widely used for the production of PA. Preparation of PA from *B. anthracis* cultures requires P-3 containment facilities (13, 14). Moreover, the yields obtained are often contaminated with other protein of *Bacillus anthracis.* Production of PA in *B. subtilis* is low because of degradation of the protein by proteases secreted by the organism (15). Purification of PA from baculovirus-vector infected in insect cells gives low yields (16). Initial attempts to purify PA from *E. coli* were not very successful because of low expression and extensive degradation of the protein (5). Expression in *E. coli* was improved by subcloning the gene in pQE30 vector systems, but the protein started aggregating and forming inclusion bodies (17, 18), requiring denaturing conditions for purification. To protect PA from cellular proteases, signal sequence of the outer membrane protein A (Omp A) of *E. coli* was added to the 5′ end of PA gene. This diverts PA to the periplasmic space (19), but purification was still laborious involving

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0006-291X/01 $35.00

ammonium sulphate precipitation, hydroxyapatite chromatography, ion-exchange, and gel filtration chromatography. Another problem was that subsequent chromatography steps involved intermediate dialysis steps. As a result it took as long as 1 week to get 0.5 mg protein from 1 liter of culture. In this paper, we present a rapid and efficient strategy for purification of recombinant PA under nondenaturing conditions. The purification process involves just two chromatographic steps viz. ion exchange and hydrophobic interaction chromatography, designed in such a way that eluate from the preceding step can be applied directly without intermediate treatment of the sample. Using this approach, 3 mg of protective antigen can now be purified to homogeneity from 1 liter culture in just 6 h.

MATERIALS AND METHODS

Expression of anthrax protective antigen. The plasmid pMS1 (19) carrying PA gene under the control of bacteriophage T7 promoter, has a Omp A signal sequence attached to the 5′ end of the gene. The plasmid was transformed into *E. coli* BL21(DE3) competent cells. The cells carrying the plasmid were grown at 37°C in LB broth with 100 μg/ml ampicillin and 0.01% $MgSO_4$ at 250 rpm. When A_{600} reached 0.8, IPTG was added to a final concentration of 0.5 mM to induce the expression of recombinant protein. Following induction, the cells were allowed to grow at 30°C for 3 h and then harvested by centrifugation at 6000*g* for 15 min at 4°C.

Preparation of periplasm. The pellet obtained from 1 liter culture was resuspended in 100 ml of 20% sucrose solution (30 mM Tris, 1 mM EDTA pH 8.0 and 1 mM PMSF) and incubated on ice for 10 min. The cells were harvested by centrifugation and resuspended in ice-cold 5 mM $MgSO_4$ containing 1 mM PMSF and 20 mM benzamidine. The cells were incubated again on ice for 10 min and then harvested at 10,000*g* for 10 min. The supernatant was collected and processed for purification of PA.

Ion exchange chromatography. The protein was purified on AKTA-FPLC using anion exchange Resource Q column (Pharmacia). The periplasm was filtered through a 0.22-μm filter and brought to a final concentration of 20 mM Tris pH 8.0. The sample was loaded on the column, which was then extensively washed with equilibration buffer (20 mM Tris, 1% glycerol, 1 mM β-mercaptoethanol, and 1 mM EDTA) till there was no change in absorbance at 280 nm. The protein was eluted with a 200-ml linear gradient of 0 to 250 mM NaCl in the same buffer. Fractions of 3 ml each were collected and were analyzed on 12% SDS–PAGE. Fractions containing PA were pooled.

Hydrophobic interaction chromatography. To further purify PA to homogeneity, hydrophobic interaction chromatography was done. Solid ammonium sulphate was slowly added to 70% saturation to the pooled fractions and the precipitation allowed for 2 h at 4°C. The precipitated protein was harvested by centrifugation at 12,000*g*. The pellet was resuspended in 20 mM Tris pH 8.0 containing ammonium sulphate at 50% saturation. The same buffer was used to equilibrate phenyl sepharose (Sigma) column before loading the sample and washing it later to remove unbound protein. Protein was eluted from the column with a 20-ml decreasing gradient of ammonium sulphate. Fractions of 1 ml each were collected, analyzed on a 12% SDS–PAGE, and those containing PA were pooled. For storing the purified protein at −70°C, buffer was changed to 10 mM Hepes, pH 7.0 containing 50 mM NaCl, using Centricon 30 kDa cut-off membrane.

Mammalian cell culture. Macrophage-like cell line J774A.1 was maintained in RPMI 1640 medium containing 10% heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Chinese hamster ovary (CHO) cell line was maintained in EMEM medium supplemented with nonessential amino acids, 25 mM Hepes (pH 7.4), gentamicin (50 μg/ml), and 10% heat-inactivated FCS.

Cytotoxicity assay. J774A.1 cells were plated at a density of 10^5 cells/ml in 96-well tissue culture plates and were grown from 80 to 90% confluence. At the start of the experiment, spent medium and detached cells were removed by aspiration and replaced with RPMI containing 1 μg/ml LF and varying concentration of PA obtained at different stages of purification. The cells were incubated for 3 h at 37°C in a humidified CO_2 incubator. All experiments were done in triplicates. After 3 h cell viability was determined with 3-(4,5-dimethylthiazol-2-yl), -5-diphenyltetrazolium bromide (MTT) dye, as described previously (6).

Quantitation of PA. The fold purification of PA at different stages was determined by calculating the amount of protein required to kill 50% of J774A.1 cells (EC_{50}) when incubated with LF (1 μg/ml) at 37°C. Protein concentrations were measured by Bradford method using USB protein determination reagent.

cAMP response of CHO. The CHO cells were plated in 24-well plates and grown to confluence. To begin the experiment, old media was replaced with H199 medium containing EF (1 μg/ml) and indicated concentration of PA. After incubation for 2 h at 37°C, intracellular cAMP was measured using nonradioactive Biotrak cAMP EIA kit (Amersham Pharmacia).

Binding of PA to cell surface receptors. Receptor binding assay was performed with J774A.1 cells plated in 12-well plates using the radioiodinated PA (purified from *E. coli* and *B. anthracis*), as described earlier (8). In brief, the cells were cooled and incubated at 4°C with radioiodinated PA (1 μg/ml). The cells were later washed four times with cold HBSS and solubilized in 0.5 ml of 100 mM NaOH. The counts were taken in a gamma counter.

In vitro cleavage of PA and its binding to EF/LF. To study the binding of LF/EF to PA in solution, PA was cleaved with trypsin (1 ng per μg of PA) for 30 min at 30°C in 25 mM Hepes, 1 mM $CaCl_2$ and 0.5 mM EDTA. Trypsin was inactivated with 1 mM PMSF and the nicked PA was incubated with LF/EF (1 μg/ml) for 15 min in 20 mM Tris, pH 9.0 containing 2 mg/ml Chaps. The samples were analyzed on a nondenaturing 5–10% gradient gel.

Polyclonal antisera. Female BALB/c mice were immunized at 6–8 weeks of age with 20 μg of PA purified from *E. coli* and emulsified in Freund's adjuvant. The intramuscular injections were given three times at 3-week intervals. Mice were bled 2 weeks after each vaccination and the serum was stored at −20°C until use.

ELISA for anti-PA antibodies. 96-Well microtiter plates were coated overnight with 10 μg/ml of *E. coli*-purified PA diluted in 50 mM carbonate/bicarbonate buffer, pH 9.6. The plates were blocked with 1% BSA (in the same buffer) for 6 h and washed with PBS-0.1% Tween. Serial dilutions of the test sera were then incubated on the plate for 6 h at room temperature. The plate was thoroughly washed and goat anti-mouse IgG antibody conjugated with HRP was added. After incubating the plate for 2 h, the plates were again washed. TMB was added to the plate and the plate was read at 450 nm using a microplate reader.

In vitro lethal toxin neutralization assay. Pooled sera from the last boost was serially diluted and incubated with 1 μg PA (from *B. anthracis*) for 1 h to allow neutralization to occur. The antiserum-toxin mixture was added to the cells along with 1 μg/ml LF. After 3 h of incubation MTT was added.

RESULTS AND DISCUSSION

Protective antigen is highly susceptible to proteases. Extensive degradation of the protein by cellular proteases has been the major cause of low yields in the

TABLE 1

Purification of PA from *E. coli*

Fraction	Volume (ml)	Amount of protein[b] (mg/ml)	Activity (EC_{50})[c]	Purification (fold)[d]
Cell lysate[a]	60	2	50	1
Periplasm	10	1.1	8	6.25
Resource Q	30	0.5	3	16.6
Phenyl sepharose	3	1	0.03	1667

[a] Cell lysate was prepared from one liter culture.

[b] Protein concentration was determined by Bradford method using USB protein determination reagent.

[c] EC_{50} is defined as the concentration of PA required along with 1 μg/ml LF to kill 50% J774A.1 cells. After 3 h of incubation cell viability was determined by MTT dye. The results are means from three different experiments.

[d] Purification fold was determined by dividing the EC_{50} of cell lysate with the EC_{50} for fractions obtained from different columns.

earlier followed protocols. Expressing protective antigen along with a signal sequence diverted the protein to the periplasm of cells. The signal sequence gets cleaved during the course of transport. This proves advantageous, as the mature protein does not have any attached tag.

Purification of PA. For the purification of anthrax protective antigen, first of all periplasm containing PA was separated from cytoplasmic proteins including proteases. This not only removes the damaging contaminants early but also results in rapid isolation of the protein, reduction of the sample volume, and six-fold (Table 1) purification of the protein. Protective antigen was then purified from the periplasmic extract of *E. coli* BL21(DE3) cells.

High versatility and high resolving power of ion exchange chromatography made it an ideal choice to achieve further purification of PA. pI of anthrax protective antigen is 5.5 and the protein is most stable in the pH range from 7.4 to 8.0. The periplasmic extract containing the protein was loaded onto a resource Q column and eluted with a very shallow gradient of NaCl. Fractions eluting from the column at 60 mM NaCl concentration were analyzed by SDS–PAGE and Western blotting (Fig. 1). Protective antigen eluted from the ion exchanger 17-fold purified (Table 1).

Ion exchange fractions containing protective antigen were pooled and precipitated with ammonium sulphate for no more than 2 h. Protective antigen precipitates out at 70% saturation of ammonium sulphate. The pellet was resuspended in Tris buffer containing ammonium sulphate at 50% saturation, applied to phenyl sepharose column, and eluted with a decreasing gradient of ammonium sulphate. Protective antigen eluted from the column at the end of the gradient as a homogeneous preparation (Fig. 1). The fractions containing PA were pooled. For storage of the protein, buffer was changed to Hepes using Centricon. The purity achieved after hydrophobic interaction chromatography was 1660-fold as compared to the cell lysate. Homogeneity of the purified protein was further confirmed by running it on two-dimensional electrophoresis (Fig. 2). Having purified the recombinant protein to homogeneity from *E. coli* cultures, further experiments were then done to study the functional and biological activity of the purified protein.

Binding of PA to cell surface receptors. The first step in the intoxication of cells by anthrax toxin is the binding of protective antigen to its receptors on the surface of host cells. To study the binding of the purified protein to the cell surface receptors, radiolabeled PA was allowed to bind to the cells in cold binding medium. The cells were washed to remove unbound protein and were then solubilized with NaOH. Radioactive counts associated with the cells were measured to determine the amount of radiolabeled protein bound to receptors on the cells. It was observed that protec-

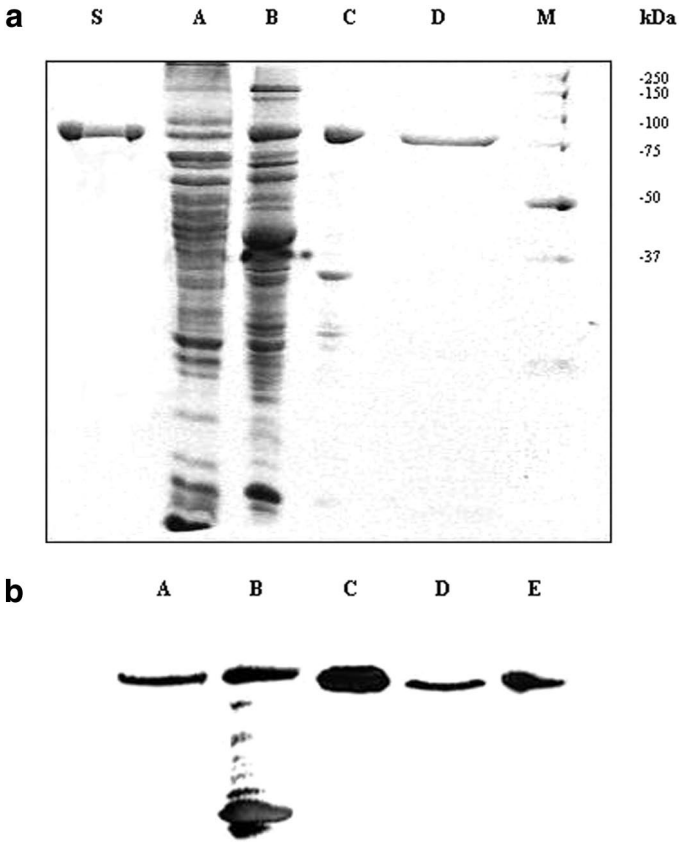

FIG. 1. Purification of *E. coli*-expressed PA. (a) Proteins analyzed on 12% SDS–PAGE gel and stained with Coommasie blue S A B C D M kDa. (b) Western blot of PA protein developed with polyclonal rabbit PA antibody. Lanes: S, PA purified from *B. anthracis;* A, cytosolic preparation of cells expressing PA; B, periplasmic profile of cells expressing PA; C, protein after passing through Resource Q anion-exchange column; D, PA after purification on phenyl-sepharose column; and M, molecular weight marker (in kDa).

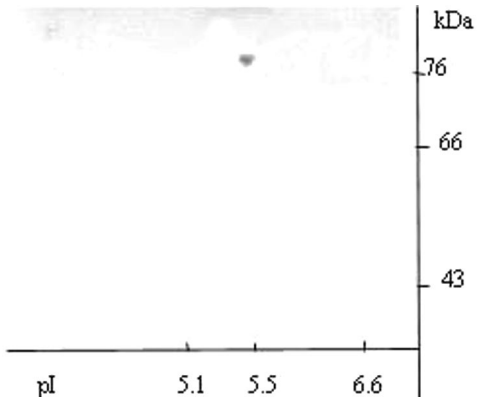

FIG. 2. 2D profile of purified PA. The purified protein was subjected to 2-dimentional electrophoresis. The 2-D profile shows PA as a single spot (83 kDa, pI 5.5) indicating that it is a homogeneous preparation.

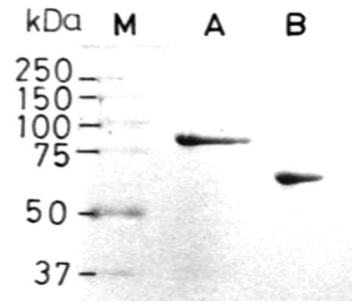

FIG. 3. Proteolytic cleavage of PA. 1 μg of *E. coli* purified-PA (lane A) was allowed to get cleaved by 1 ng of trypsin for 30 min at 30°C in 25 mM Hepes, 1 mM $CaCl_2$, and 0.5 mM EDTA. The digested protein sample (lane B) migrated as a 63 kDa band on 12% SDS–PAGE.

tive antigen purified from *E. coli* and *B. anthracis* showed comparable binding to cell surface receptors (Table 2).

Proteolytic cleavage of PA. Following binding of PA to cell receptor, the next step in the intoxication process is proteolytic cleavage of PA to 63 kDa fragment, PA63, which has an exposed high affinity binding site for LF/EF. This proteolytic cleavage of PA can be mimicked in solution by treating PA with trypsin. Results in Fig. 3 show that *E. coli*-purified PA can be cleaved with trypsin to give the 63 kDa protein.

Binding to LF/EF. Cleavage by proteases like trypsin and furin, exposes the binding site for EF and LF on PA63.

The binding of LF/EF with PA can be easily detected on a native PAGE as a slow moving band. Figure 4 shows that PA purified from *E. coli* is capable of binding both to LF and EF.

These results showed that PA is functionally active. Next step was to study the biological activity of *E. coli*-purified protein and compare it with that of PA purified from *B. anthracis.* Protective antigen is the central component of anthrax toxin complex that facilitates the entry of the catalytic moieties, LF and EF, into susceptible cells where they exert their toxic effects. Biological activity of PA was studied on two different cell lines: J774A.1 and CHO.

Cytotoxicity assay. J774A.1 macrophage-like cells are susceptible to lysis by lethal toxin. Cytotoxicity assay performed on these cells, by incubating various concentrations of PA with 1 μg/ml of LF showed that PA purified from *E. coli* was biologically active and was as potent as its *B. anthracis* counterpart in causing cell death (Table 3).

cAMP assay. Anthrax edema factor is an adenylate cyclase that elevates the intracellular cAMP levels in the host cells (20). Treatment of CHO cells with *E. coli*-purified PA along with EF caused elongation of these cells. This is peculiar response of CHO cells to agents that increase intracellular cAMP levels. Measurement of intracellular cAMP concentration of CHO cells was done and it was observed that cAMP concentration rise proportionally to the dose of PA when EF is kept constant. The activity of PA purified from

TABLE 2

Binding of PA to Cell Surface Receptors[a]

Protein	Specific binding of PA[b]
Recombinant PA from *E. coli*	9200 ± 454
Native PA from *B. anthracis*	9885 ± 523

[a] J774A.1 cells plated in 12-well plates were incubated with 1 μg/ml radio-iodinated PA (purified from *E. coli* and *B. anthracis*) at 4°C. The cells were later washed with cold HBSS, solubilised in 100 mM NaOH, and then radioactive counts were taken.

[b] Specific binding is the difference between the mean counts per minute (± standard error) of triplicate samples bound with or without a 100-fold molar excess of nonradioactive PA.

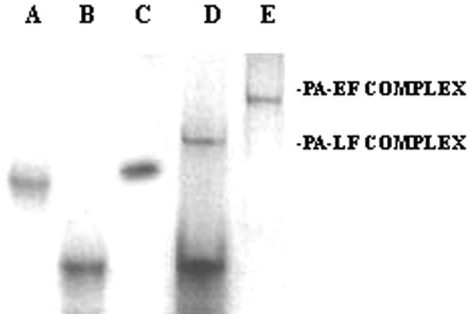

FIG. 4. Binding of recombinant PA to LF/EF in solution. 1 μg of trypsin-nicked PA was incubated with LF or EF for 15 min and the samples were then analysed on a nondenaturing 4–10% gradient gel. The gel was then stained with Coomassie blue. Lane A, PA purified from *E. coli.* Lane B, anthrax lethal factor. Lane C, anthrax edema factor. Lane D, PA–LF complex. Lane E, PA–EF complex.

TABLE 3

Cytotoxicity Produced in J774A.1 Cells by Anthrax Lethal Toxin

	Percentage viability[a]	
PA (μg/ml)	*E. coli*-purified PA	*B. anthracis*-purified PA
0.001	100	100
0.01	60	62
0.1	15	12
1	5	5

[a] J774A.1 cells were incubated with 1 μg/ml of LF along with the indicated concentrations of PA (purified from either hosts) for 3 h at 37°C. Cell viability was determined by MTT assay. Untreated cells or cells treated with just one toxin component (PA or LF) had 100% viability. The data values are representative of three different experiments done in triplicates.

the hosts, *E. coli* and *B. anthracis*, was comparable (Table 4).

Immunological response. Having established that the recombinant protein was comparable to the native *B. anthracis* protein in terms of biological activity, we proceeded to compare the immunogenicity of the two proteins. Intramuscular injections of purified recombinant protective antigen emulsified with Freund's adjuvant elicited comparable immunological response in animals. Anti-PA titers (in ELISA) against recombinant protective antigen were 1:28,000 and that against native *B. anthracis* protein were 1:30,000. To show that the antibodies in the sera of immunized animals could protect against anthrax toxin, we performed an *in vitro* anthrax toxin neutralization assay. Serial dilutions of antiserum obtained from mice (immunized with recombinant PA) was allowed to preincubate with protective antigen from *B. anthracis* for 1 h before adding it along with LF/EF to the susceptible cells. An antibody dose-dependent protection curve was obtained. It was observed that 1:18,000 dilution of antiserum (against recombinant PA) could protect 50% cells from the action of anthrax lethal toxin. Likewise preincubation of PA with the antiserum protected CHO cells from the elongation response of anthrax edema toxin. 1:15,000 dilution of antiserum caused 50% inhibition of CHO cell elongation. These results are of significance in light of previous reports that have shown that antiserum against PA can provide significantly high passive protection against anthrax. Also, the use of antitoxin as an adjunct with bactericidal antibiotics is recommended for effective treatment against PA.

Protective antigen is a necessary immunogen in the current human vaccine against anthrax. However, in addition to PA, trace amounts of other culture-derived proteins including LF and EF are also present that contribute to side effects of the vaccine. Immunization with the human vaccine can induce local pain, edema, and erythrema. Moreover, a total of six immunizations within 18 months, followed by yearly boosters, are required. The efficacy of the vaccine is affected by the challenge strain of anthrax, vaccine batch variation, and the adjuvant used. The veterinary vaccine is a suspension of spores from a noncapsulated, toxinogenic, Sterne strain of *B. anthracis*. But, like the human vaccine, the veterinary vaccine often causes adverse reactions, observed both after primary application and revaccination. Thus there is a need to develop high resolution purification methods that will increase the possibility of developing PA-based new generation vaccine against anthrax that have lower reactogenecity, improved efficacy and reduced immunization schedule.

Expression and purification of anthrax protective antigen from hosts like *Bacillus subtilis*, baculovirus, etc., was not successful because of the low yields obtained (15, 16). Expression was improved in *E. coli* (using T5 promoter), which produces large amounts of recombinant protective antigen in aggregate form as inclusion bodies (17). After solubilization of the inclusion bodies in strongly denaturing buffer (guanidium hydrochloride or urea), the protein was immobilized on Ni-NTA column to remove the denaturant. The conformational integrity of protein, subsequently eluted from the column, was not studied. Studies with other proteins have shown that reconstitution of conformation-dependent epitopes of a denatured protein is a very complex problem. For example, researchers in pursuit of producing correctly folded, renatured N-terminal domain of human acetylcholine receptor (21), tried several expression vectors and renaturation conditions including immobilization of protein on Ni-NTA column to remove denaturant, its gradual removal by dialysis and rapid dilution of denaturant. Correct conformation (as

TABLE 4

cAMP Response of CHO Cells to Edema Toxin

	Intracellular cAMP formed (nmol/mg)[b]	
PA (μg/ml)[a]	*E. coli*-purified PA	*B. anthracis*-purified PA
0.001	0.038	0.032
0.01	0.45	0.35
0.1	1.58	1.15
1	4.90	4.52

[a] Cells plated in 96-well tissue culture plates were treated with media containing EF (1 μg/ml) and indicated concentration of PA from either of the sources. After 2 h, intracellular cAMP of the treated cells was determined using Biotrak cAMP EIA kit. Protein content of the wells averaged 7 μg. Cells treated with EF or PA alone did not show an increase in cAMP levels and had 0.028 and 0.039 nmol of cAMP/mg of CHO cell protein.

[b] All values are representative of three different experiments done in triplicates with mean standard error being less than 5%.

judged by binding of mAb against conformation-dependent epitopes) was not obtained with any of these strategies. Finally, cyclodextran was used to remove the denaturant and guide the correct folding of the protein. Clearly, unless the reconstitution of conformation-dependent epitopes is achieved, the apparently refolded protective antigen cannot be expected to provide protection. Another drawback with recombinant protective antigen purified by Ni-NTA affinity chromatography is the attached 6×-Histidine tag. Any tag attached to the mature protein must be removed before the protein can be considered as a potential vaccine.

The work reported here focuses on purification of protective antigen to homogeneity under a rapid and nondenaturing condition to ensure that there is no loss of activity and the conformational integrity of epitopes is not disturbed. The results presented here may be used for development of a safe and efficacious recombinant vaccine against anthrax.

ACKNOWLEDGMENTS

The authors would like to thank DBT, Government of India for providing financial support for the study. Both Nidhi Ahuja and Praveen Kumar received financial aid from UGC, Government of India.

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