Appl Biochem Biotechnol (2009) 157:554–561
DOI 10.1007/s12010-008-8309-x

Functional Expression of *Bacillus anthracis* Protective Antigen in *E. coli*

Fatemeh Vahedi · Mojtaba Sankian · Mahmoud Mahmoudi

Received: 24 March 2008 / Accepted: 27 June 2008 /
Published online: 25 July 2008

Abstract The protective antigen (PA) of *Bacillus anthracis* is a potent immunogen and an important candidate vaccine. In addition, it is used in monitoring systems like enzyme-linked immunosorbent assay to assess antibodies against PA in immunized subjects. The low level of PA production in *B. anthracis* and the difficulty of separating it from other bacterial components have made the researchers do different studies with the aim of producing recombinant PA (rPA). In this study, to produce rPA as a recombinant protein vaccine, the partial sequence of protective antigen of *B. anthracis*, amino acids 175–764, as a potent immunogenic target was inserted in pET21b(+). This is a prokaryotic plasmid that carries an N-terminal T7.tag sequence. The integrity of constructed plasmid was confirmed using restriction enzyme mapping. rPA was expressed after induction with isopropyl β-D-1-thiogalactopyranoside in *Escherichia coli* BL21. Purification of rPA was done with an affinity system using anti T7.tag antibody. Electrophoresis and Western blotting confirmed the specificity of the expressed protein. BALB/c mice were immunized with obtained PA protein and evaluation of specific immunoglobulin G antibodies against PA in sera using Western blotting method and showed that rPA is immunogenic. The challenge of immunized mice with virulent strain of *B. anthracis* showed that rPA is functional to protect against pathogenic strain.

Keywords *Bacillus anthracis* · Protective antigen (PA) · Expression · Immunogenicity

Introduction

Anthrax is a well-known historical disease and was one of the first to be described in association with its causative organism, *Bacillus anthracis* [1]. Although, understanding the molecular basis of its pathogenesis has begun only in recent years [2].

F. Vahedi (✉)
Razi Vaccine & Serum Research Institute, Ahmadabad Street, P.O. Box 91735-174, Mashhad, Iran
e-mail: f.vahedi@rvsri.ir

M. Sankian · M. Mahmoudi
Immunology Research center, Mashhad University of Medical Science, Mashhad, Iran

The principal virulence factor of *B. anthracis* is a multi-component toxin secreted by the organism that consists of three separate gene products designated as protective antigen (PA), lethal factor (LF), and edema factor (EF). The genes encoding these toxin components (*pag*, *lef*, and *cya*, respectively) are located on a 184-kb plasmid designated pX01 [3].

Protection against anthrax infection is associated with the humoral immune response directed against PA [4]. PA is the dominant antigen in both natural and vaccine-induced immunity to anthrax infection [5]. It is also essential for host cell intoxication in combination with either LF or EF, producing lethal toxin, or edema toxin, respectively. PA contains the host cell receptor-binding site and facilitates the entry of the toxin complex into the host cell [4].

The currently available human vaccines against *B. anthracis* are far from ideal; they are expensive to produce, require repeated doses, and may invoke transient side effects in some individuals. These vaccines consist of alum-precipitated material from cultures of toxigenic, non-encapsulated strains of *B. anthracis*. Immunization with these vaccines occasionally causes local pain, edema, and erythema and requires several boosters [6]. Both native and recombinant PA preparations elicit high antibody response. PA protein therefore can be used to develop an effective acellular recombinant vaccine against anthrax [7]. The crystal structure of PA has been previously determined and its effective utilization has been shown [2].

In addition, PA is used in monitoring systems like enzyme-linked immunosorbent assay to assess raised anti-PA antibodies in immunized humans and animals [8].

Different studies have been done to produce PA by inserting the PA gene into different bacterial species such as *Escherichia coli*, *Bacillus subtilis*, and *Bacillus brevis* and subsequently expressed, previously [9–13].

Furthermore, the yeast *Saccharomyces cerevisiae* has been used to produce a recombinant 63-kDa form of *B. anthracis* protective antigen [14].

The primary objective of this study was to prepare a recombinant PA for the development of a vaccine candidate against *B. anthracis* that may be more efficacious and less reactogenic. Our approach has been to insert the PA gene into pET21b(+) vector capable of expressing PA after induction with IPTG in *E. coli* cells. Demonstrated in this paper are the expression of PA in *E. coli* and the antigenicity and the immunogenicity of expressed PA in mice.

Materials and Methods

Strains, Plasmids, Media, and Reagents

The avirulent *B. anthracis* strain 34 F2 (Sterne) was obtained from the Razi vaccine and Serum Research Institute (Mashhad, Iran). *E. coli* strain DH5α (Stratagene) was used for initial cloning, sequencing, and maintenance of different DNA fragments. For recombinant protein production, a prokaryotic expression vector pET21b (Novagene) was used. The recombinant plasmid was transformed into *E. coli*, BL21 (DE3) pLysS (Stratagene) as host strain. Luria–Bertani (LB) broth or LB agar was supplemented when required with 100 μg/ml ampicillin. Anti-PA monoclonal antibody was purchased from KOMA (Korea). All chemicals were obtained from Merck (Germany) and all of the enzymes from Fermentas (Lithuania) or Cinnagen (Iran) Companies.

Extraction of Total DNA

A simple and rapid method was used for total DNA extraction. Briefly, 200 μl of heat inactivated bacterial culture of *B. anthracis* strain 34 F2 (Sterne) was vortexed vigorously

and then was frozen in liquid nitrogen. The sample was thawed and vortexed again. These steps were repeated three times, and then the sample was centrifuged at 12,000×*g* for 1 min. The obtained supernatant was used as DNA source directly.

Primers Design

The PA gene (GenBank accession number Ay91578) was chosen as target. The forward primer, pePa5 (5′AAGCTTACCATGGTTCCAGACCGTGAC3′) and the reverse primer, pcPa3 (5′CTCGAGCTTCAATTACCTTATCCT3′), containing recognition sites for *Xho*I and *Hin*dIII, were used for amplification of PA gene. The primers were synthesized by MWG Biotec, Germany.

Amplification of PA Gene, Insert Segment Into Vector pET21b(+) Plasmid

Polymerase chain reaction (PCR) reaction was performed in 50 μl volume containing 500 ng of template DNA, 1 μM of each primer, 2.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates, 1× PCR buffer and 2.5 U of *pfu* DNA polymerase (Fermentas). The following conditions were used for amplification: hot start at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min. The program followed by a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in 1% agarose gel in Tris/borate/ethylenediaminetetraacetic acid buffer and visualized by ethidium bromide staining on UV transilluminator. The PCR product was purified from the agarose gel by high pure PCR product purification kit (Roche Diagnostic) according to the manufacturer's instructions. The purity of eluted PCR product was checked by NanoDrop ND-100 spectrophotometer. For further analyses and before sequencing, the PCR product was subjected to restriction enzyme analyses.

Construction of pET21b+ PA Plasmid

Amplified PA gene was purified from low-melting agarose gel and digested by *Hin*dIII and *Xho*I restriction enzymes as manufacturer's protocol. pET21b(+) vector was prepared for ligation after amplification in *E. coli* DH5α bacteria, digestion with *Hin*dIII and *Xho*I enzymes and dephosphorylation using calf intestine phosphatase. The prepared insert was ligated into treated pET21b(+) vector using T4 DNA ligase at 16°C overnight. *E. coli* DH5α and *E. coli* BL21 (DE3) plysS competent cells were prepared by calcium chloride method and were used for transformation. The transformed bacteria were selected by screening the colonies on media containing ampicillin. The suspected colonies were further analyzed by restriction enzyme digestion, PCR, and sequencing to ensure of plasmids integrity.

Induction of PA Expression with IPTG

A 5-ml culture of the recombinant BL21 λDE3 was prepared in LB containing 100 μg/ml ampicillin. The culture was incubated at 37°C with shaking to an OD_{600} of 0.5. The culture was split into 2× 2.5 ml cultures. IPTG was added to one of the 2.5-ml culture for a final concentration of 1 mM. The other culture was saved as control. Both cultures were incubated while shaking at 37°C. Samplings were done by removing 0.5 ml of cultures at 0, 1, 2, 3, and 4 h. Removed samples were centrifuged immediately at 10,000×*g* for 30 s, and the supernatant was removed. The cell pellets were frozen at −20°C immediately. To lysis the frozen bacteria, the samples were thawed, and each cell pellet was resuspended in

500 μl of lysis buffer (50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM Imidazole, pH=7.8). The resuspended samples were frozen by deeping in liquid nitrogen and then thawed at 42°C. This step was repeated three times. The samples were centrifuged at maximum speed for 1 min at 4°C to pellet insoluble proteins. The supernatants were transferred to fresh tubes on ice.

Analyzing Samples by “”SDS-PAGE and Western Blotting

Extracts of *E. coli* transformed with plasmid pET21b+ PA were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels to check for expression of PA. Sample aliquots from both cell pellets and culture media were examined by Laemmli method [15]. The gels were stained with Coomassie Blue or silver nitrate. For blotting, proteins were then electro-transferred from the gel to nitrocellulose membrane to be prepared for immunoblotting. Nitrocellulose membranes with attached proteins were blocked in 1% bovine serum albumin (BSA) for 3 h to prevent non-specific binding. The blocked membranes were then washed three times and anti-PA monoclonal antibody (4 mg/ml, KOMA) at a 1:2,000 dilution was used, and then, membranes were incubated for 3 h at room temperature. The membranes were washed again three times and anti-mouse immunoglobulin/horseradish peroxidase conjugate was added. After 1 h incubation at room temperature and washing, the substrate, diaminobenzidin as the chromogen, was employed for visualizing. Brown color developed where the primary antibody recognized protein bands.

Purification of rPA Protein

rPA protein was purified from 100-ml induced culture using T7.Tag Affinity purification kit (Novagen) as manufacturer’s protocol. Immunoaffinity purification was done with column procedure and T7.Tag antibody agarose. Purified proteins were analyzed on SDS-PAGE gels again. Protein concentration was determined using the modified Bradford assay [16]. BSA of 0, 2, 4, 6, 10, 15, and 20 μl (1 mg/ml) were pipetted into wells of a 96-well plate. Twenty microliters of samples were poured subsequently. Forty microliters of Bradford reagent, consisting of 100 mg Coomassie Blue G-250 in 50 ml 95% ethanol, 100 ml 85% (*w*/*v*) phosphoric acid in 850 ml distilled water, was added into all wells containing standard or sample to bring the final volume to 200 μl, and absorbencies were read at 595 nm without any prior incubation. The standard curve of absorbance versus micrograms protein was plotted to find the linear regression line.

Immunization and Challenge of Mice

To assess the humoral immune responses induced by purified rPA, 4- to 5-week female BALB/c mice in groups of ten each received 0.5-ml dose containing 20 μg rPA on days 0, 30, and 60, intramuscularly (i.m.), with Freund’s complete adjuvant, Freund’s incomplete adjuvant, and free, respectively. One group was sham-inoculated with sterile phosphate-buffered saline (PBS). Ten days after the last immunization, mice were infected intraperitoneally (i.p.) with 10^3 spores of a virulent strain of *B. anthracis* (C2). Death of animals was recorded for 10 days after challenge.

All mice were subjected to bleeding before each injection or challenge from the tail vein, and the sera were stored in −20°C. The sera were subjected to Western blotting as described in the previous section.

Results and Discussion

Construction of Recombinant Plasmid, pET21b+ PA

The expression vector pET21b(+) was used to PA producing construct, pET21b+ PA. The constructed plasmid was amplified in *E. coli* DH5α that is a recombination-deficient amber-suppressing strain. For the expression of PA gene, *E. coli* BL21 DE3 was used. BL21 DE3 is used for high expression of genes cloned into expression vectors containing the bacteriophage T7 promoter such as pET21b(+) [17]. *Xho*I and *Hin*dIII cloning sites were chosen for insertion. Therefore, T7.tag (33 aa) was incorporated in N-terminal of expressed protein. This vector can express an 11-amino acid T7.tag at the N terminus. These additional amino acids increase the size of the expressed protein about 5 kDa.

Ligation of the PA gene insert into pET21b+ and subsequent transformation of *E. coli* DH5α yielded amp-resistant colonies. After purification of plasmids from selected colonies, single and double digests on each plasmid with *Xho*I and *Hin*dIII determined the presence of the 1,721-bp PA gene.

Integrity of cloning were more confirmed with the amplification of ×1,700-bp fragment with pePA5, pcPA3 and T7, and pcPA3 pair primers on pET21b+ PA plasmid.

The selected expression clone was also analyzed with restriction map analyses, and its integrity was confirmed.

In addition, the purified recombinant plasmid (pET21+ PA) was sequenced. The sequencing result was confirmed by comparing with the databases and using the basic local alignment search tool software (data not shown). The sequence reported in this paper has been deposited in the GenBank database (accession number EF550209).

Expression, Purification, and Analyses of Recombinant PA Protein

It was observed that PA protein was expressed immediately after induction with IPTG. The expected recombinant fusion protein of ×64 kDa was detected after induction of the culture with IPTG, most of it was found to be localized inside inclusion bodies (pellet of cell lysate) in the cells (Figs. 1 and 2).

Expressed PA by *E. coli* BL21 was purified with an affinity system using T7 tag. PAGE and blotting results have revealed the presence of an excess band that has specificity for anti-PA. Based on previous reports, it is proposed that rPA is subjected to degradation by

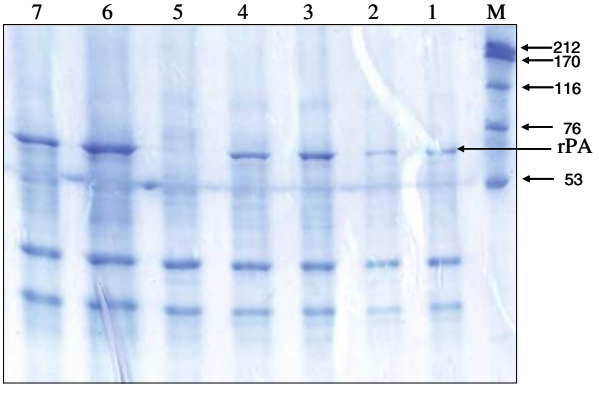

Fig. 1 SDS-PAGE (10%) for analyzing of PA expression in soluble fraction of lysate bacteria with Coomassie Blue staining. *Lane M* Molecular weight marker, *lane 1* 2 h after IPTG induction, *lane 2* 1 h after IPTG induction, *lane 3* 4 h after IPTG induction, *lane 4* 3 h after IPTG induction, *lane 5* soluble fraction from control culture, without induction, *lane 6* 20 h after IPTG induction, *lane 7* 10 h after IPTG induction. A 64-kDa band is seen in all lanes except in lane 5, without induction

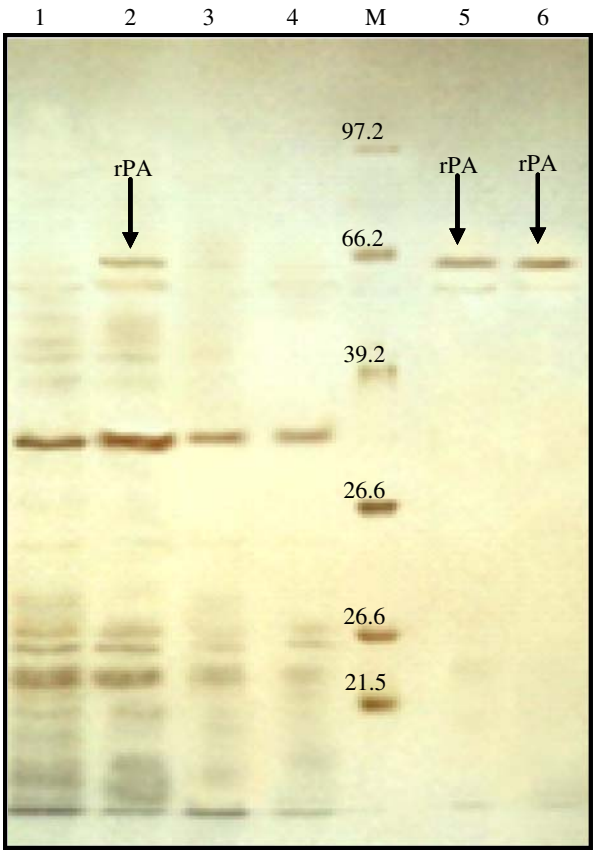

Fig. 2 SDS-PAGE (5%) for analyzing of PA expression in unsoluble fraction of lysate bacteria with silver nitrate staining. *Lane M* Molecular weight marker; *lane 1* unsoluble fraction of bacterial culture before induction; *lane 2* insoluble fractions of bacterial culture after induction, two excess bands are visualized (*rPA*), a major 64-kDa band and a faint 58-kDa band; *lanes 3 and 4* eluted fraction from affinity chromatography, unbound proteins; *lanes 5 and 6* purified rPA, a major 64-kDa band, and a faint 58-kDa band are visualized

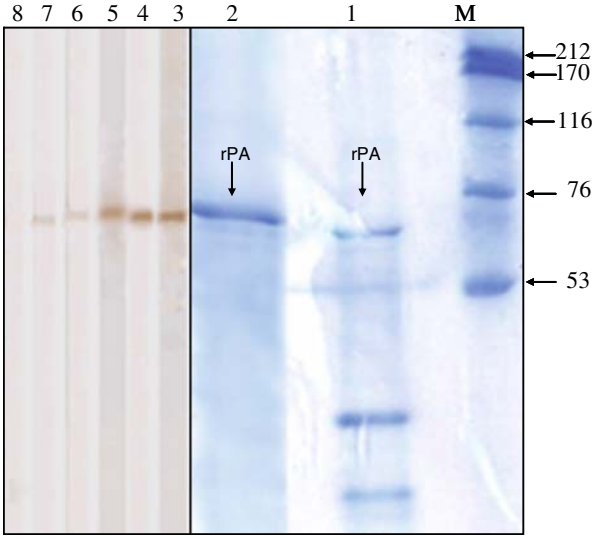

Fig. 3 Western blots of rPA protein exposed to anti-PA monoclonal antibody and sera from immunized mice. *Lane M* Molecular weight marker, *lane 1* insoluble fraction of bacterial culture with rPA, *lane 2* pure rPA, *lanes 3–5* blotted samples of purified rPA (*lane 1*) exposed to anti-PA monoclonal antibody, *lanes 6–7* pure rPA exposed to sera from mice immunized with rPA. The *colored band* shows the presence of specific antibodies against rPA, *lane 8* pure rPA exposed to sera from negative control mice

bacterial proteases [18, 19]. To further support this theory, we showed that the lighter band became stronger when increasing the incubation period of cells.

Because the degraded band has been reacted with anti-T7 tag, fragment has been removed from the C-terminal of PA.

The PA protein was purified from 2- and 16-h-induced cultures using T7.Tag affinity chromatography under denaturing conditions followed by on-column renaturation of the protein before elution. Purified proteins were analyzed using SDS-PAGE to assess their purity. These proteins were subjected to Western blotting, and two bands of 64- and 58-kDa sizes showed specificity to anti-PA antibody (Fig. 3).

At the point of harvest, it was possible to achieve yield of 0.002 mg/ml (15%) in 2-h-induced and 0.0039 mg/ml (21%) in 16-h-induced cultures.

Leppla and Vodkin [20] cloned the *pag* gene in *E. coli* to express PA. Subsequently, several colonies producing PA were identified; however, the level of protein expression was extremely low [21]. Furthermore, PA was isolated from *E. coli*, but it was severely degraded and was inactive functionally [22]. In 1999, researchers in India using *E. coli* were able to purify recombinant PA of correct size and functionally active [23]. Anthrax toxin was originally purified from the supernatant of *B. anthracis*; yet, these preparations were contaminated with other host proteins [24]. PA production from *B. subtilis* or a protease-deficient *B. subtilis* host did not yield large quantities of PA [25]. Production of stable PA from *E. coli* has exhibited limited success.

Immunogenicity of rPA

The sera of immunized mice were subjected to Western blotting. Western blot revealed the presence of antibodies against PA in the sera of groups that received rPA (Fig. 3).

Ten days after challenging the mice with virulent *B. anthracis* bacteria, the rate of survival was determined. The immunized mice with rPA showed a survival rate of 70%. But in the negative group, the survival rate was low (20%) and differed significantly from immunized groups ($P<0.01$). The result of this experiment shows that rPA has immunogenicity with protection effect against pathogenic strain of *B. anthracis*.

In this study, we could obtain an immunogenic and semi-stable rPA. Further study should be done to optimize the rPA production to achieve higher yield.

References

1. Weiss, M. M., Weiss, P. D., & Weiss, J. B. (2007). *American Journal of Public Health*, *97*, 1945–1951. doi:10.2105/AJPH.2006.102749.
2. Petosa, C., et al. (1997). *Nature*, *385*, 833–838. doi:10.1038/385833a0.
3. Vahedi, F., et al. (2005). *Iranian Journal of Immunology*, *3*, 134–140.
4. Brossier, F., Guidi-Rontani, C., & Mock, M. (1998). *Comptes Rendus des Seances de la Société de Biologie et de ses Filiales*, *192*, 437–444.
5. Mourez, M., et al. (2003). *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 13803–13808. doi:10.1073/pnas.2436299100.
6. Baillie, L. W. (2006). *Journal of Applied Microbiology*, *101*, 594–606. doi:10.1111/j.1365-2672.2006.03112.x.
7. Splino, M., et al. (2005). *Annals of Saudi Medicine*, *25*, 143–149.
8. Grunow, R., et al. (2007). *Vaccine*, *25*, 3679–3683. doi:10.1016/j.vaccine.2007.01.056.
9. Rhie, G. E., et al. (2005). *FEMS Immunology and Medical Microbiology*, *45*, 331–339. doi:10.1016/j.femsim.2005.05.008.

10. Baillie, L. W., Johnson, M., & Manchee, R. J. (1994). *Letters in Applied Microbiology*, *19*, 225–227. doi:10.1111/j.1472-765X.1994.tb00949.x.
11. Chauhan, V., et al. (2001). *Biochemical and Biophysical Research Communications*, *283*, 308–315. doi:10.1006/bbrc.2001.4777.
12. Gupta, P., Sahai, V., & Bhatnagar, R. (2001). *Biochemical and Biophysical Research Communications*, *285*, 1025–1033. doi:10.1006/bbrc.2001.5282.
13. Gwinn, W., et al. (2006). *Protein Expression and Purification*, *45*, 30–36. doi:10.1016/j.pep.2005.04.009.
14. Hepler, R. W., et al. (2006). *Vaccine*, *24*, 1501–1514. doi:10.1016/j.vaccine.2005.10.018.
15. Laemmli, U. K. (1970). *Nature*, *227*, 680–685. doi:10.1038/227680a0.
16. Bradford, M. (1976). *Analytical Biochemistry*, *72*, 248–254. doi:10.1016/0003-2697(76)90527-3.
17. Miroux, B., & Walker, J. E. (1996). *Journal of Molecular Biology*, *260*, 289–298. doi:10.1006/jmbi.1996.0399.
18. Novak, J. M., et al. (1992). *The Journal of Biological Chemistry*, *267*, 17186–17193.
19. Friedman, T. C., et al. (1995). *Archives of Biochemistry and Biophysics*, *316*, 5–13. doi:10.1006/abbi.1995.1002.
20. Vodkin, M. H., & Leppla, S. H. (1983). *Cell*, *34*, 693–697. doi:10.1016/0092-8674(83)90402-6.
21. Sharma, M., et al. (1996). *Protein Expression and Purification*, *7*, 33–38. doi:10.1006/prep.1996.0005.
22. Ahuja, N., Kumar, P., & Bhatnagar, R. (2001). *Biochemical and Biophysical Research Communications*, *286*, 6–11. doi:10.1006/bbrc.2001.5337.
23. Gupta, P., Waheed, S. M., & Bhatnagar, R. (1999). *Protein Expression and Purification*, *16*, 369–376. doi:10.1006/prep.1999.1066.
24. Quinn, C. P., et al. (1988). *The Biochemical Journal*, *252*, 753–758.
25. Hambleton, P., & Turnbull, P. C. (1990). *Advances in Biotechnological Processes*, *13*, 105–122.