Production and Purification of Protective Antigen of *Bacillus anthracis* and Development of a Sandwich ELISA for its Detection

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ABSTRACT

Anthrax, a zoonotic disease caused by *Bacillus anthracis* is important for biowarfare as well as public health point of view. The virulence factors of *B. anthracis* are encoded by the two plasmids, pXO1 and pXO2. Protective antigen (PA), an 83 kDa protein encoded by pXO1 along with lethal factor (LF, 90 kDa) or edema factor (EF, 89 kDa), makes the anthrax toxin responsible for causing the disease. Current detection and diagnostic systems for anthrax are mostly based on PA, a potential biomarker of *B. anthracis*. The objective of the present study was to produce and purify the PA for development of a sandwich ELISA for its detection. In this study, pYS5 plasmid containing the full PA gene was transformed into an 8 proteases deficient *Bacillus anthracis* host BH480. The PA was produced under shake flask conditions and purified using the gel filtration chromatography. The reactivity of PA with rabbit and mouse anti-PA antibodies was confirmed by Western blotting. The antibodies were purified and used for the development of a sandwich ELISA for detection of PA. The detection sensitivity of ELISA was found to be 3.9 ng/ mL PA.

Keywords: *Bacillus anthracis*; Protective antigen; purification; ELISA

1. INTRODUCTION

Bacillus anthracis, a Gram-positive, spore-forming bacterium causes anthrax in higher mammals including human. Depending on the route of exposure, anthrax can be of three types viz. cutaneous, gastrointestinal or inhalational[1]. The inhalational (pulmonary) anthrax is considered to be the most severe form among the three due to its high mortality rate. *B. anthracis* forms the spores which are highly resistant under extreme harsh environmental conditions and persist for many decades[2]. *B. anthracis* spores can easily be aerosolised and disseminated. Therefore, *B. anthracis* is considered as a category 'A' bio-threat agent[3,4].

B. anthracis contains two large plasmids. pXO1 and pXO2 encoding the toxigenicity and pathogenicity factors, respectively. The pXO1 encodes protective antigen (PA), edema factor (EF) and lethal factor (LF)[5], whereas the pXO2 encodes poly-γ-D-glutamic acid capsule having anti-phagocytic property. PA, LF and EF are individually non-toxic, however, LF and EF in association with PA make the lethal toxin and edema toxin, respectively[6].

PA is the central factor of the tripartite complex, and hence has always been the major target for the development of anthrax vaccine or diagnostic assays[7-10]. The PA protein is secreted in medium during the growth of *B. anthracis in vitro* but the amount of PA is very low and also have small amount of EF and LF[11]. Hence, due to contamination of PA with other toxins it can't be used for diagnosis purpose. Moreover,

handling of *B. anthracis* for production and purification of native PA is risky. To overcome this problem. PA gene has been transformed into various hosts for the production of recombinant PA such as *Esherichia coli*[12-14], *Bacillus subtilis*[15,16], *B. anthracis*[17,18], *Bacillus brevis*[11], *Baculovirus* and *Vaccinia* Virus[19], and *Pseudomonas fluorescens*[20]. However, each expression system has some limitations. *B. anthracis* and *B. subtilis* produce various proteases in the growth medium leading to degradation of PA.

The early diagnosis of anthrax increases the likelihood of survival and recovery of the patient by timely treatment. Most of the serodiagnostic assays for anthrax are based on detection of antibodies against PA or LF[21,22]. However, these methods can be employed only after development of antibody response in patient post infection. For the early diagnosis, anthrax toxins are the best target due to early secretion of toxin in the course of disease before detectable bacteremia or immune response[8,23]. In an earlier developed monoclonal antibody based capture sandwich ELISA, as low as 1 ng/ml of PA could be detected from the serum of Guinea pig and rabbit[24]. In another study, 10 pg/ml of PA could be detected in artificially spiked human blood samples by surface plasmon resonance[8] Hence, direct detection of toxin can provide a timely diagnosis compared to serology or culture-dependent methodologies. In the present study, pYS5 plasmid containing full PA gene was transformed into an 8 proteases deficient *B. anthracis* host BH480. The PA was produced and purified from the recombinant host and used for the production of polyclonal antibodies in rabbit and mouse. The antibodies were further used for the development of a sandwich ELISA for detection of PA.

2. MATERIALS AND METHODS

2.1 Strains and Plasmids

B. anthracis Ames BH480 strain[25] (with the genotype pXO1[-], pXO2[-], Spo0A^{-} (GBAA_4394), NprB[-] (GBAA_0599), TasA[-] (GBAA_1298), Cam[-] (GBAA_1290), InhA1^{-} (GBAA_1295), InhA2^{-} (GBAA_0672), MmpZ[-] (GBAA_3159), CysP1^{-} (GBAA_1995), VpR[-] (GBAA_4584), and the pYS5 plasmid containing original pagA promoter from pXO1, including 162 bp of *B. anthracis* DNA upstream of the PA start codon used in this study were obtained from Institute of Genomics & Integrative Biology, Delhi. The pYS plasmid was transformed into dam[-] and dcm[-] *E. coli* SCS110 strain. The non-methylated plasmid of SCS110 was then transformed into the electro-competent *B. anthracis* BH480 strain, which is avirulent and sporulation defective strain[25].

2.2 Reagents and Chemicals

All culture media for protein production were from Difco (USA). The Superdex 200 used for gel filtration column was purchased from GE Healthcare (Sweden). The adjuvant, HRP conjugated secondary antibodies and all other chemicals were purchased from Sigma Chemical Co. (USA) unless indicated otherwise.

2.3 Ethical Statement

All animal care and experimental procedures were conducted as per the approved protocols of Institutional Animal Ethics Committee (IAEC) of DRDE (Reg. No.37/Go/C/1999/CPCSEA) and Institutional Biosafety Committee (Protocol no. IBSC/12/BT/AKG/22). All the experimental mice were maintained in pathogen free facility.

2.4 Production and Purification of PA from *B. anthracis*

The recombinant PA from *B. anthracis* was expressed and purified as described earlier with minor modifications[26]. Briefly, a 50 mL of LB broth containing 25 µg/mL of kanamycin was inoculated with *B. anthracis* BH480. After 8 h incubation at 37 °C, 5% (v/v) of the culture was further inoculated into 500 mL of super broth (SB) medium supplemented with 0.5% bicarbonate and incubated for 16 h at 37 °C. After centrifugation at 15317 x g at 4 °C for 20 min, the supernatant containing PA was collected.

The PA protein was precipitated with 60 % ammonium sulphate at 4 °C and recovered by centrifugation at 1860 x g for 20 min. Ammonium sulphate precipitation concentrates the protein as well as some media components also. The media components interfere in column during protein purification, therefore, media components were removed by dialysis. The pellet was re-suspended in Phosphate buffer saline containing 20 % glycerol followed by dialysis in the same buffer. Further, the dialysed protein was purified using gel filtration chromatography[27]. The Superdex 200 column was pre-equilibrated with 50 mM phosphate buffer (pH 7.4) at a constant linear flow rate of 50 cm/h. Dialysed protein was loaded to the pre-equilibrated column. The purified protein was eluted from the column using 50 mM phosphate buffer containing 20% glycerol. Elutes were analysed by SDS-PAGE

and stored at -80 °C until further use.

To increase the stability of the PA protein in the buffer, 20% glycerol was added in the buffer as the glycerol increases the stability as well as inhibits the protein aggregation in vitro[28,29].

2.5 Production and Characterisation of anti PA Rabbit and Mouse Polyclonal Antibody (pAb)

New Zealand white rabbits and BALB/c mice were used for production of anti-PA polyclonal antibodies as described elsewhere[30]. Final bleeding of rabbit and mice was carried out on day 60. The sera were separated from blood and used in Western blot and ELISA.

The purified PA was run on 12 % SDS-PAGE and transferred on PVDF membrane and incubated with 1:2000 dilution of anti-PA mouse or rabbit serum as described earlier[30]. The membranes were incubated with anti-mouse or anti-rabbit IgG HRP conjugate (1:10000) for 1 h at 37 °C, and developed with 3,3′-Diaminobenzidine/H_2O_2 substrate solution for 2 min at room temperature.

Antibody titre of rabbit and mouse sera was evaluated by indirect ELISA as described earlier[31]. Sera of rabbit and mouse collected before immunisation were used as control. The antibody titer was expressed as reciprocal of the endpoint dilution. For the development of ELISA antibodies were purified using protein G column and the purity was determined on SDS-PAGE.

2.6 Development of Sandwich ELISA for Detection of PA

A sandwich ELISA was developed for detection of PA as described earlier[32]. Briefly, ninety-six-well ELISA plates were coated overnight with purified rabbit anti-PA antibodies (200 ng/well) in carbonate-bicarbonate coating buffer (0.06 M Na_2CO_3 and 0.14 M $NaHCO_3$), pH 9.5 at 4 °C. Plates were then washed three times with 0.1% tween-20 in PBS, pH 7.4 (PBS-T) followed by blocking with 5% skimmed milk protein (SMP) at 37 °C for 1 h. After washing with PBS-T, different concentrations of purified PA starting from 200 ng to 0.19 ng/well were added and incubated at 37 °C for 1 h. The plates were again washed and incubated with 200 ng/well anti PA mouse polyclonal antibody in 1% SMP (in PBS) for 1 h at 37 °C. Anti PA mouse polyclonal antibodies were used as a revealing antibodies which were probed with rabbit anti-mouse HRP-conjugate (1:4000) TMB substrate was added after incubation at 37 °C for 1 h and kept at 37 °C for 10 min. The plate was read at 450 nm after adding 50 µl of 2.5 N H_2SO_4 to each well. The samples were tested in triplicate.

3. RESULTS

3.1 Production and Purification of PA

In this study, we used the *B. anthracis* strain BH480, which is asporogenic, lacks both the virulence plasmids, and is deleted for 8 extracellular proteases. The strain was transformed with PA gene containing pYS5 plasmid.

A protein band of ~83 kDa on the SDS PAGE of supernatant confirmed the expression of PA (Fig. 1). The ammonium sulphate precipitated protein was analysed by
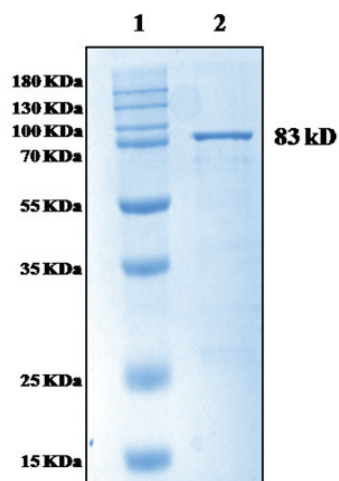
Figure 1. SDS-PAGE profile of expressed PA. Lane 1, MW marker; lane 2 Culture supernatant containing PA.

SDS-PAGE. No band was observed at ~83 kDa in supernatant, while an intense band of PA was observed in the pellet (Fig. 2(a)). The precipitated protein was dialysed and purified by gel filtration column and a single band of ~83 kDa was found on SDS-PAGE (Fig. 2(b)). The elutes containing PA protein were pooled and the concentration was checked by BCA assay. A final protein concentration of 1.0 mg/ mL was made and stored at -80 °C for further use.
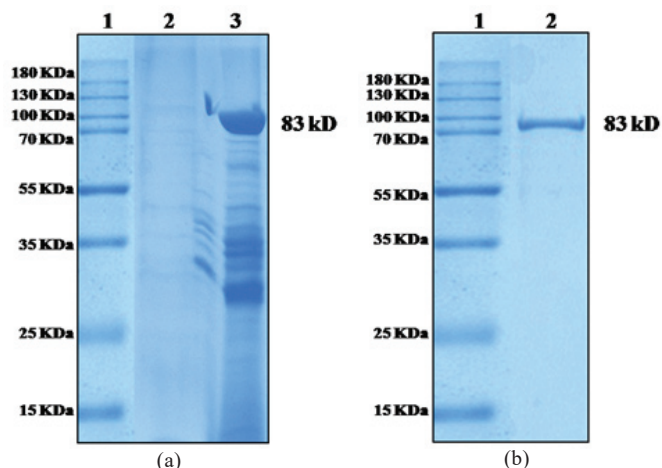

Figure 2. SDS-PAGE profile for ammonium sulphate precipitated and purified protein. (a) Ammonium sulphate precipitation of PA. Lane 1, MW marker; lane 2, Supernatant after ammonium sulphate precipitation; lane 3, Pellet of ammonium sulphate precipitate. (b) Purified recombinant PA. Lane 1, MW marker; lane 2, Purified protein.

3.2 Production and Characterisation of Anti PA Rabbit and Mouse Polyclonal Antibodies

The PA raised high levels of specific antibody titer. Western blot was performed to check the reactivity of the anti-PA rabbit and mouse sera with purified recombinant PA of *B. anthracis*. The anti-PA rabbit and mouse sera gave reactivity with the PA of *B. anthracis* in Western blot (Fig. 3).
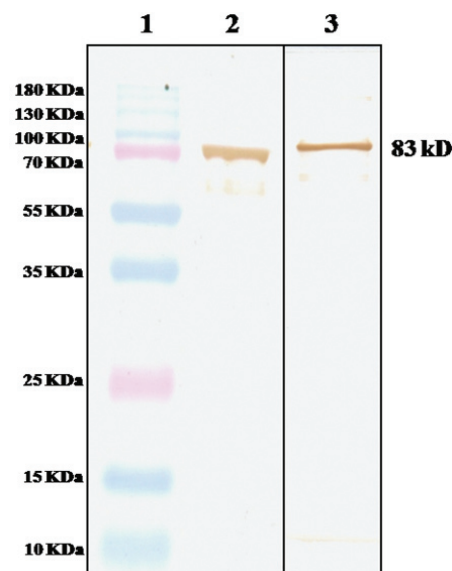
The antibody titres of rabbit and mouse sera were

Figure 3. Reactivity of anti-PA sera with purified PA by Western blot. Lane 1, MW marker; lane 2, anti-PA rabbit sera; lane 3, anti-PA mouse sera.

determined by ELISA. The antibody titre of rabbit serum was 1: 256000 with PA in indirect ELISA. The antibody raised in mouse serum also exhibited a titre of 1: 256000.

3.3 Development of Sandwich ELISA

For the development of sandwich ELISA, the antibodies were purified from the serum and checked by SDS-PAGE. In the antigen capture ELISA, rabbit antibodies were used for capturing and mouse antibodies were used as revealing antibodies. An antigen concentration from 200 ng to 0.19 ng per well was tested. The OD *vs* PA concentration profile is shown in Fig. 4. PA as low as 3.9 ng/mL could be detected by sandwich ELISA (Fig. 4).

4. DISCUSSION

B. anthracis is listed as a 'Category A' biothreat agent by the Center of Disease Control and Prevention (CDC), USA. Surveillance of *B. anthracis* being an agent of bio-warfare as well as public health importance in several agrarian
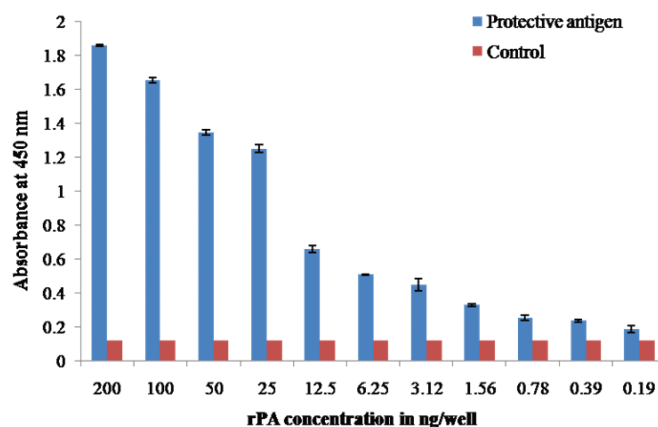

Figure 4. Detection sensitivity of recombinant PA by sandwich ELISA using polyclonal antibodies.

countries is very important for timely detection of epidemics[33]. Apart from worldwide cases, in India, both sporadic cases and outbreaks of anthrax are reported regularly from many states[4].

There are well established antibiotic regimens for the treatment or recovery of anthrax in human as well as animals. However, anthrax vaccination is the most effective method of prevention before exposure. Anthrax vaccine adsorbed (AVA) is the licensed vaccine for people at high risk of exposure. However, empirically it is not possible to use anthrax vaccine for mass vaccination. Hence, early diagnosis is very important to post exposure prevention methods to curtail the spread of disease as well as for patient management[4].

Purified recombinant protein is required for diagnostic use. In the present work, pYS5 plasmid containing PA gene was transformed in *B. anthracis* BH480, which lacks 8 protease enzymes and prevents proteolytic cleavage of PA in the growth media. To enhance the production of PA and its stability, various manipulations have been done in *B. anthracis*. Non-sporogenic, protease-deficient, avirulent BH445[18] and BH460[34] strains were developed. *B. anthracis* strain BH480 is 8 protease deficient strain for production of PA in native conditions[25]. For the production of PA in *Bacillus*, various shuttle systems have also been constructed. The shuttle vector pYS5 from *Bacillus – E. coli* was constructed to clone a recombinant PA in the host cell[35].

The culture supernatant was precipitated by ammonium sulphate to concentrate the PA. The precipitated protein was re-suspended in PBS buffer containing 20 % glycerol and dialysed before purification. A single step chromatography purification process was employed for purification of PA. The purified protein yielded a single band with expected molecular weight of ~83 kDa on SDS-PAGE (Fig. 2(b)).

The purified protein was used to generate polyclonal antibodies in rabbit and mouse. The sera reacted with PA in Western blot. The antibody titer was checked by indirect ELISA. The purified antibodies were used for development of sandwich ELISA using rabbit antibodies for capturing and mouse antibodies for revealing. The detection limit of developed ELISA was 3.9 ng/mL in dilution buffer (Fig. 4). Previously, anti-PA monoclonal antibodies were used to develop the sandwich ELISA and as low as 1 ng/ml of PA could be detected from the serum of rabbit and Guinea pigs[24]. By surface plasmon resonance (SPR), as low as 10 pg/ml of PA was detected from the human serum artificially spiked with PA[8].

The antibodies and the sandwich ELISA can be further used for detection of PA in the clinical serum samples. Earlier, it has been reported that PA in the infected sera could serve as a reliable marker of infection[36]. It was observed that there is a direct correlation between the quantitative of PA and level of bacteraemia in the sera of both rabbit and guinea pig models[36].

In conclusion, the recombinant PA was produced from *B. anthracis* and purified using gel filtration chromatography in this study. Sandwich ELISA developed using the polyclonal antibodies against the purified PA exhibited its potential for the detection of PA. Hence, the ELISA can further be used for

detection of PA in the clinical samples.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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