Production of *Bacillus anthracis* Recombinant Protective Antigen in *Escherichia coli*

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Abstract

Over the century anthrax has been a fundamental model for the studies of infectious diseases. The anthrax toxin consists of three proteins namely, protective antigen (PA), lethal factor, and edema factor. These are produced by the Gram-positive bacterium, *Bacillus anthracis*. Current vaccines against anthrax consist of protective antigen as the primary component. Production of recombinant PA of *Bacillus anthracis* was carried out by fermentation process in *E. coli* at 37^{0}C, pH 7.2 for 8 hours. The dissolved oxygen level was maintained at 30-40 % using inlet air as well as pure oxygen whenever required. Expression of the recombinant PA was induced with 1 mM IPTG and further grown for five hours before harvesting. Expression of PA in *E. coli* yielded an insoluble protein aggregating to form inclusion bodies. The inclusion bodies were solubilized in 8 M Urea and the protein was purified under denaturing conditions using nickel nitrilotriacetic acid (Ni-NTA) affinity chromatography. The recombinant *E. coli* resulted in cell dry weight of about 8.43 g per litre of culture. This translates into an expression yield of about 36.46 mg of the PA per litre of culture. The purity of the recombinant PA was checked by SDS-PAGE analysis and reactivity of this protein was determined by Western blotting and ELISA. Thus, the recombinant PA may be used for further development as a diagnostic reagent as well as vaccine candidate against anthrax.

Keywords : Protective antigen; *Bacillus anthracis; Escherichia coli;* Fermentation

Introduction

The bacterium, *Bacillus anthracis* is the causative agent for the disease called anthrax (Dixon *et al.,* 1999). Recently, *B. anthracis* has attracted attention as an agent for bioterrorism (Inglesby *et al.,* 1999; Lane *et al.,* 2001). The toxic effects of B. anthracis are primarily due to the "anthrax toxin," which consists of a complex of three proteins: protective antigen (PA), edema factor (EF), and lethal factor (LF). The three proteins of the toxin are encoded by plasmids pXO1 and the polyglutamate capsule is encoded by pXO2. PA is the most extensively characterised component of anthrax toxin. The molecular weight of PA is 83 kDa. Protective antigen (PA) of *Bacillus anthracis* is the central moiety of the anthrax toxin complex and it elicits antibody response useful for serodiagnosis of the disease. The primary immunogenic component of the human vaccine is the protective antigen (PA) (Hambleton and Turnbull, 1990). It is also the main immunogen of the cell-free vaccine against anthrax. Immunization with the human vaccine can induce local pain, edema, and erythema, and frequent boosters are required (Brachman *et al.,* 1962). The current licensed vaccine consists of a cell-free filtrate of *Bacillus anthracis* culture grown to maximize PA content, the major immunogen (Ahuja *et al.,* 2001; Ramirez *et al.,* 2002). There is considerable under reporting of the disease largely because of lack of adequate microbiological facilities and test systems for diagnosis of anthrax (Kumar *et al.,* 2000).

In India, only conventional identification methods are being used to diagnose the disease. Serological and molecular diagnostic methods are not in use due to the paucity of standardized reagents and there is no human vaccine in developing countries like India for prevention of the disease. *Escherichia coli* is the most commonly used host for heterologous protein production. Using expression vectors in batch and fed-batch cultivations, recombinant proteins have been successfully expressed in recombinant E. coli. However, many of these proteins accumulated in the form of insoluble biologically inactive inclusion bodies (Lee, 1996; Riesenberg and Gutkhe, 1999). Thus, the volumetric productivity of a recombinant protein is proportional not only to the final cell density but also to the specific yield (the amount of product formed per unit cell mass).

Therefore, in the present study efforts have been made for production of recombinant PA in *E. coli* to achieve higher yields and to utilize this purified protein for detection of anti-PA antibodies. This protective antigen could be used as a reagent for the timely diagnosis of anthrax as well as for further vaccine studies.

Materials and Methods

Bacterial cultures

Recombinant Escherichia coli was obtained from Dr Rakesh Bhatnagar (Jawaharlal Nehru University, New Delhi). The PA gene along with 6X histidine coding sequence was ligated into pQE30 vector. The SG13009 (pREP4) *E. coli* host cells were transformed with this vector (Gupta *et al,* 1999). Ampicillin (100µg/ml) and Kanamycin

(25µg/ml) was used for preparation of seed culture and fermentation. Stock culture was preserved at -80 ^{0}C in 30 % glycerol.

Shake flask experiments

Cells were grown in shake flask cultures in LB medium. Recombinant gene expression was induced using 1mM IPTG at an optical density (OD 600) of 0.6. Cells were harvested 5 h post induction. The composition and preparation of the defined medium for shake flask cultures has been described previously (Tripathi *et al.,* 2007). The carbon source in shake flask experiments with defined medium was 10 g/ l glycerol.

Medium and Inoculum preparations

The composition and the preparation of the medium were essentially same as described previously (Tripathi *et al.,* 2007) along with the following minor deviations. The glycerol concentration for the batch culture was 10 g/l. For the preparation of 8 litre batch medium tryptone, yeast extract, sodium chloride, $(NH_4)_2HPO_4$, KH_2PO_4 and $MgSO_4.7H_2O$ were dissolved in distilled water in the bioreactor, the pH was adjusted to pH 7.2 using solid NaOH, and the solution was sterilized for 30 min at 121°C. The feeding solution (glycerol 300 g/l) was sterilized separately for 15 min at 121°C. The first preculture (50 ml LB medium in 250 ml shake flask) was inoculated with 1 ml of frozen stock and incubated on a rotary shaker at 37°C for 8 h. Following this, 200 ml defined medium in 1 litre shake flasks was inoculated with the first preculture (1 % v/v) and incubated on a rotary shaker at 37°C for 16 h. Prior to inoculation, the optical density of the secondary precultures was determined and inoculation of the bioreactor was carried out such that the batch culture started with an OD 600 of 0.1 with approximately 160 ml of preculture for 8 - l of batch medium.

Recombinant *E. coli* cultivation in Bioreactor

The main cultivations were carried out at 37°C in a bioreactor of working volume of 10-litre (BioFlo 3000, New Brunswick Scientific, USA) equipped with extensive analytical devices and a process control system for control and data-sampling (BioCommand Plus, New Brunswick, USA). The initial batch culture conditions were as follows: initial culture volume-8l, air flow-8 l /min, stirrer speed-200 rpm. Thermal mass flow controller was used for mixing air and oxygen. Dissolved oxygen was maintained at 30-40% of air saturation by increasing stirrer speed and aeration rate. If required the inlet air was enriched with pure oxygen. The pH was maintained at pH 7.2 by addition of aqueous ammonia (25% v/v). All controls were carried out by the process control system of the bioreactor. After consumption of the initial nutrient, as indicated by an increase of the dissolved oxygen concentration, the fed-batch phase was started by using DO stat feeding . Foam was detected by an automated foam control system and suppressed by the addition of the Antifoam A (Sigma,USA). . The culture was induced with IPTG to a concentration of 1mM. Following induction the culture was further grown for 5 hours before harvesting. The fermentation broth was centrifuged at 6000 rpm for 15 minutes at 4^{0}C.

Purification of Protective Antigen

As PA was mainly localized in the inclusion bodies, the protein was purified under denaturing conditions.The pellet was resuspended in Ni-NTA buffer, pH 8(1:10 w/v) containing 8 M urea, 100 mM sodium phosphate, and 100 mM Tris-Cl. Cells were stirred at room temperature for 1 h. Lysate was centrifuged at 10,000g for 40 min at 4°C. The supernatant was mixed with 50% slurry of Ni-NTA resin and allowed to stir at room temperature for 45 min, and then the resin was loaded carefully into affinity chromatography column which was pre equilibrated with above buffer. The column was washed with 10 column volumes of Ni-NTA buffer (pH 6.3). The recombinant protein was then eluted with Ni-NTA buffer (pH 4.5). The fractions were analyzed on SDSPAGE, and those containing the protein of interest were pooled. The pooled samples were concentrated using lab-scale tangential flow filtration (Millipore, France) and stored in aliquots at -80°C.

Analytical methods

The off-line analysis during cultivation such as the determination of the OD 600, the cell dry weight (CDW) and the preparation of samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were carried out as described previously with minor modifications (Tripathi *et al.,* 2008). Electrophoresis was carried out using 10% SDS-polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R250 and quantification was done by BCA method using bovine serum albumin for calibration. For Western blot analysis, recombinant PA was resolved on 10% denaturing gel (SDS-PAGE), along with prestained protein molecular weight markers and further procedures were performed similar to our earlier study (Tripathi et al, 2006) using 5% Bovine serum albumin (BSA) as the blocking agent, goat anti-mouse IgG horseradish peroxidase (HRPO) as the conjugate and 3, 3'-diaminobenzidine tetra hydrochloride (DAB)-H_2O_2 as the chromogenic substrate.

Biological Activity of recombinant Protective Antigen by ELISA

The biological activity of the rPA was determined by Enzyme Linked Immunosorbent Assay (ELISA) in the similar way as described earlier (Tripathi *et al.,* 2006).Briefly, purified rPA protein was diluted to 10 g/ml in 0.1 M carbonate buffer, pH 9.6, and used for coating 96-well microtiter plates (100 l/well) at 37 °C for 1 hour. Further processing was carried out using 5% Bovine serum albumin (BSA) as the blocking agent, serial two-fold dilutions of immune serum against rPA as primary antibody, goat anti-mouse IgG horseradish peroxidase (HRPO) as the conjugate and Ortho phenyl Diamine (OPD) as substrate.

Results and Discussion

Production of rPA in fermentor

Production of recombinant proteins in shake flask cultures is in general performed using LB medium to support cell growth and protein production. However, for recombinant protein production with higher yield, cells are required to grow on complex or defined medium to allow the application of batch or fed-batch mode. To investigate the productivity of recombinant *E. coli* on these two different media, cells were grown in shake flask cultures either on LB or on the defined medium used for fermentation and the

production of the recombinant protein was induced with 1mM IPTG. The analysis of the total cell protein of *E. coli* producing the recombinant PA on LB or on defined medium by SDS-PAGE revealed that the recombinant protein was produced in the form of inclusion bodies. Fig.1 shows the real time profile of fermentation process for production of recombinant PA.

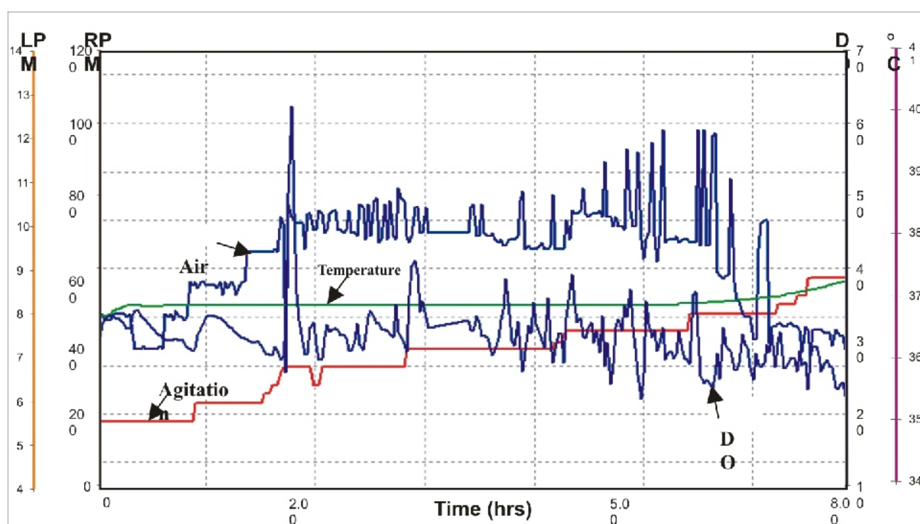

Fig.1 Real time profile of fermentation for production of rPA protein

To increase the production of rPA fermentation process was adopted. E. coli cells harbouring recombinant PA gene were grown in media containing tryptone, yeast extract and glycerol to produce large quantities of rPA. The cultures at OD of 9.31 (~3.50 g/l dry cell weight) were induced with 1 mM IPTG and grown for another 5 h, and the batch was terminated at OD of 24.11 (~8.61 g/l dry cell weight).OD versus time profile is shown in Fig.2. With increase in the biomass, the O2 demand also increased during fermentation phase. Rise in pH and DO values indicate that the carbon source or one of the substrates is limiting. The maximum amount of rPA protein was obtained after 5-hour post induction. The final cell density of about 8.61 g dry cell weight/l was reached at ~8 hours. The composition of the growth media is crucial for enhancing product formation as well as acetate reduction. Acetate is not produced when glycerol is used as a carbon source and high cell densities may be achieved relatively easily using glycerol. Since the primary goal of fermentation research is cost-effective production of recombinant products, it is important to develop a cultivation method that allows the maximization of yields of the desired product. The fermentor provides the opportunity to control multiple factors for maintaining an optimal culture environment, biomass production and protein yield. Some of the factors that can be maintained during fermentation include the ability to control the pH of the culture, to provide effective aeration, control of foaming and also to maintain culture sterility.

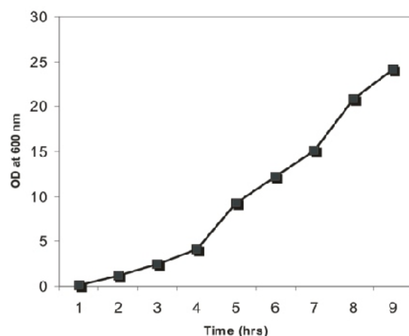

Fig.2: OD600 vs time during batch fermentation

Purification and characterization of recombinant PA protein

Expression of heterologous protein in *E. coli* allows its rapid and economical production in large amounts. However, some new problems arise, especially in case of inactive protein synthesis and inclusion body (IB) formation. Inclusion bodies need to be solubilized by denaturants, and target protein has to be refolded to its native biologically active conformation. In spite of this disadvantage and various scientific efforts to obtain soluble target protein in host *E. coli* strain, inclusion body formation is still considered as a convenient and effective way in recombinant protein production. Expression of target protein more than 30% of the total cellular protein, easy isolation of the inclusion bodies from cells, lower degradation of the expressed protein, high level of target protein homogeneity in inclusion bodies, and possibility to reduce the number of purification steps are usually indicated as the main advantages of inclusion body formation (Singh and Panda 2005). As the localization experiment showed that the recombinant protective antigen is found in insoluble fraction, the rPA was purified by Ni-NTA metal affinity chromatography. The IBs were solubilized in 8M urea containing buffers and purified by affinity chromatography column under denaturing conditions. The protein could be eluted out with the pH gradient. The final yield of rPA was ~36.46 mg/l of fermentor culture (Table 1). The final yield of rPA after fermentation increased about 18 times in comparison with commonly used shake flask culture with LB medium.

Purified rPA was separated on denaturing SDS-PAGE gel and found to migrate with an appropriate molecular mass of ~83 kDa (Fig. 3). Purified protein was characterized by Western blotting analysis using hyper

Table 1: Production characteristics of rPA in shake-flask and fermentor.

Media	Culture Condition	Dry Weight (g/l)	Protein (mg/l)
Luria bertani broth	Shake flask	1.13	~2.40
Defined media	Shake flask	2.68	~4.16
Defined media	Fermentor	8.61	~36.46

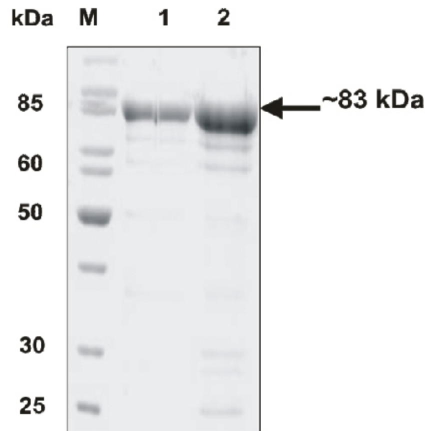

Fig.3 - SDS-PAGE analysis of rPA protein after Ni-NTA affinity purification. Lane 1, Molecular Weight Marker (kDa); lane 2, 3 Purified rPA protein.

immune sera, raised in mice against rPA (Tripathi *et al.,* 2006). The results revealed the reactivity with this protein (result not shown).

Biological activity of rPA protein in ELISA

The rPA was expressed and purified with the objective of its use as a diagnostic reagent for detection of PA antibodies as well as a vaccine candidate. For this, we tested the rPA with hyper immune serum raised in mice with different dilution by using indirect plate ELISA. The PA-specific antibody titer was found to be more than 204800 (Fig.4). The results showed that rPA expressed by *E.coli* is able to elicit PA-specific antibody responses.

Conclusions

The methods presented in this study allow production of large quantity of anthrax recombinant protective antigen for the purpose of diagnosis as well as prophylaxis of anthrax. For cost-effectiveness, it is important to enhance protein production by development of efficient fermentation process coupled with appropriate purification methods. This protein may effectively be utilized for detection of anti-PA antibodies in serum samples of anthrax patients for the timely management and control of the disease.

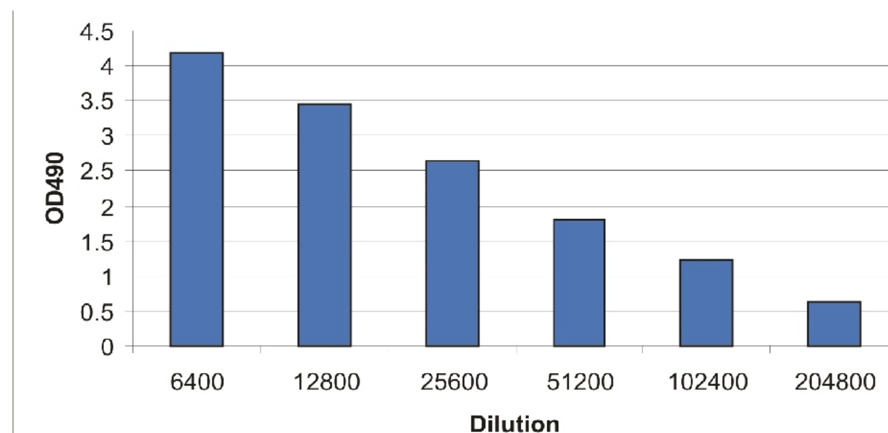

Fig.4: Endpoint titre of HIS against rPA in ELISA

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