Enhanced production and purification of recombinant surface array protein (Sap) for use in detection of *Bacillus anthracis*

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Abstract

Surface array protein (Sap) can be an important biomarker for specific detection of *Bacillus anthracis*, which is released by the bacterium during its growth in culture broth. In the present work, we have cloned and expressed Sap in *Escherichia coli*. The culture conditions and cultivation media were optimized and used in batch fermentation process for scale up of Sap in soluble form. The recombinant Sap was purified employing affinity chromatography followed by diafiltration. The final yield of purified protein was 20 and 46 mg/l of culture during shake flasks and batch fermentation, respectively. The protein purity and its reactivity were confirmed employing SDS–PAGE and Western blot, respectively. The antibodies raised against purified Sap were evaluated by Western blotting for detection of Sap released by *B. anthracis*. Our results showed that the Sap could be a novel marker for detection and confirmation of *B. anthracis*.

Keywords Anthrax · *Escherichia coli* · Bioreactor · Purification · Surface array protein

Introduction

Anthrax is a potentially fatal zoonotic disease and humans get infection on coming in contact with the affected animals. *Bacillus anthracis*, the causative agent is recognized as the most potential biological warfare agent (Goel 2015). Besides, anthrax is also a public health problem in several agrarian countries. Therefore, it is important to develop simple technologies for detection of *B. anthracis*.

Surface layers (S-layers) are generally the outermost components of several bacteria. However, in *B. anthracis* S-layers are highly patterned ultra structure layers found beneath the capsule made up of poly-D-glutamic acid (Mesnage et al. 1999). *B. anthracis* harbors two types of S-layer proteins, viz., extracellular antigen-1 (EA1) and surface array protein (Sap), which are encoded by the chromosomal genes *eag* or *sap*, respectively (Kern et al. 2012; Mesnage et al. 1997). *B. anthracis* bacteria are entrapped by Sap under exponential growth phase. Later on, this Sap is replaced by EA1 when the bacteria reaches stationary phase (Candela et al.

2005). Both, Sap as well as EA are highly antigenic and immunogenic proteins (Walper et al. 2012). EA1 has been used for species specific detection of *B. anthracis* spores (Lai et al. 2003). However, later on it was found that instead of an integral component of spore, EA was a contaminant during spore preparation (Williams and Turnbough 2004). *B. anthracis* secretes Sap in growth medium under in vitro conditions, whereas other bacteria do not. Thus, Sap may be utilized as a biomarker for early detection of *B. anthracis*.

The present study was aimed at high level production of recombinant Sap antigen in *E. coli* using bioreactor, and its utility as an important biomarker for detection of *B. anthracis*.

Materials and methods

Cloning of sap gene

B. anthracis Sterne grown in Luria Bertani (LB) broth was used to extract the genomic DNA employing the method described elsewhere (Jackson et al. 1997). The surface array protein (*sap*) gene (GenBank accession no. Z36946.1) was PCR amplified using the primers, sap-F (5'-CATGGATCCGCAGGTAAAACATTCCCAG-3', the *Bam*H1 site underlined) and sap-R (5'-ATACTCGAGTTTGTGTCAGGTTT

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TGCTTCT-3', the *Xho*I site underlined). The PCR product (2355 bp) was purified and cloned in the pET32a+ expression vector (Novagen, USA) using the restriction enzymes, *Bam*HI and *Xho*I. The ligated plasmid was transformed into *E. coli* BL21(DE3).

Preliminary expression of Sap

The cultures were grown in LB broth for 16 h at 25 °C after induction with 1 mM IPTG. After 16 h of incubation at 200 rpm and 25 °C, the cultures were centrifuged and the pellet was resuspended in cell lysis buffer. The soluble fraction (supernatant) and insoluble fraction (pellet) were analyzed using anti-His antibody (Sigma, USA) in Western blot. The expression conditions were optimized for enhanced production of Sap.

Optimization of cultivation media and culture conditions for soluble over-expression of Sap

For media optimization, 1 ml of 16 h grown culture was inoculated into 50 ml of each of the five media (Difco BD, USA) with antibiotics in duplicate, i.e., LB, LB with 1% glucose, LB with 1% glycerol, LB with 0.12% MgSO_4 , and Terrific Broth (TB) in 250 ml conical flasks. After induction with 1 mM IPTG, the cultures were incubated at 25 °C for 16 h. Samples were collected for measurement of OD_{600} , wet or dry cell weight and recombinant Sap expression level.

Bioreactor studies

Recombinant Sap production was further scaled up using 5 L working volume bioreactor (Bioflo 3000; New Brunswick, USA) under optimized conditions. Bioreactor vessel containing 4.5 L of TB medium with respective antibiotic was inoculated with 5% (v/v) of 14 h grown culture. The initial bioreactor cultivation conditions were temperature, 37 °C; pH, 7.0; agitation, 100 rpm; and air, 2.5 LPM. The dissolved oxygen (DO) concentration was kept at 20–30%. The cultures were grown till mid log phase (4 h post inoculation), after which temperature was kept at 25 °C and inducer (1 mM IPTG) was added for protein expression. After 16 h, the cultures were centrifuged and pellet stored at –20 °C.

Purification of Sap using affinity chromatography and diafiltration

For purification of Sap, lysis buffer was added to the cell pellet and sonicated for 10 min (9 s on and 9 s off). After centrifugation, the supernatant was filtered through 0.45 μm membrane filter and loaded on to 5 ml pre-packed Ni-NTA affinity chromatography column (Qiagen, Germany). The protein was purified by AKTA Explorer chromatography

system at a flow rate of 1 ml/min for column equilibration, sample load, column wash and protein elution. The column was washed using wash buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 50 mM Imidazole, pH 8.0) with 10 CV. The protein was eluted in elution buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 250 mM imidazole, pH 8.0), and diafiltered with centrifugal device (50 kDa) using progressively decreasing imidazole concentration (200, 100, 50 and 0 mM).

Production of antibodies

New Zealand white rabbits weighing 800–1000 g were obtained from the animal house facility of our Institute. The animal experiments were performed according to the Institutional Animal Ethics Committee (IAEC) vide registration number 37/1999/CPCSEA. Pre-immune serum was collected from rabbits before immunization for use as control. Each rabbit was immunized subcutaneously with 200 μg of Sap in Freund complete adjuvant. Subsequently, three booster doses of Sap were given with Freund incomplete adjuvant at an interval of two weeks. After one week of last injection, the animals were test bled and serum titre was determined by plate enzyme linked immunosorbent assay (ELISA) employing Sap. Antibodies from sera were purified using 5 ml pre-packed protein G column (GE Healthcare, Sweden) and analyzed by SDS–PAGE.

Indirect ELISA for anti-Sap antibody titre

Indirect ELISA was performed to evaluate the titre of anti-Sap antibodies. Ninety-six well ELISA plates were coated with purified recombinant Sap (200 ng/well) in coating buffer (pH 9.6) and incubated for 16 h at 4 °C. After washing the plates, 300 μL of blocking buffer (5% SMP in PBS, pH 7.2) was added to each well, and incubated for 1 h at 37 °C followed by three washings with wash buffer. A 100 μL of twofold serially diluted serum was added to each well and kept for 1 h at 37 °C. After 3 washes, 100 μL /well of HRP-conjugated anti-rabbit IgG (1:10,000) was added and incubated at 37 °C for 1 h. After three washings, 100 μL of 3,3',5,5'-tetramethylbenzidine (Sigma Aldrich, USA) was added to each well to produce a color. After incubation at 37 °C for 10 min, the plates were read at 630 nm using an ELISA plate reader (BioTek Instruments Inc, USA). Pre-immune serum was used as control in the test. Each serum sample was tested in triplicate. Antibody titre was expressed as reciprocal of the end point dilution.

Detection of *B. anthracis*

For detection of *B. anthracis*, the culture was grown in 5 ml of brain heart infusion (BHI) broth for 16 h at 37 °C, 180 rpm and the samples were collected at different time

intervals. To test the specificity of the assay, other strains of *Bacillus* sp. i.e., *B. cereus*, *B. globigii*, *B. pumilus*, *B. subtilis* and *B. thuringiensis* were inoculated in 5 ml of BHI broth and samples were collected after 16 h incubation at 37 °C. The collected samples were centrifuged and culture supernatant was kept in boiling water for 10 min to inactivate the live bacteria, if any. The heat inactivated samples were tested for presence of Sap by Western blot using rabbit anti-Sap antibodies.

Results and discussion

Cloning and expression of surface array protein

The PCR product of *sap* gene (2355 bp) was cloned into pET32a + vector and transformed in *E. coli* BL21(DE3). The expression of recombinant Sap was confirmed by SDS PAGE.

Optimization of expression conditions

The inducer concentration (IPTG), induction temperature and induction time for soluble and enhanced expression of recombinant Sap were optimized. An IPTG concentration of 1 mM, induction temperature of 25 °C, and 16 h of induction time was found optimum for expression of recombinant Sap.

These optimized conditions resulted in overall higher protein production in soluble form. Previously also it has been shown that protein expression at lower temperature helps to decrease non-specific hydrophobic interactions responsible for protein folding (Vera et al. 2007).

Effect of culture media on expression of recombinant Sap

The ODs and protein expression levels varied in different media under the shake flask culture conditions. The highest OD of 2.19 was achieved in TB medium. The OD values of 1.6, 1.3, 1.4 and 1.64 were observed in LB, LB with 1% glucose, LB with 1% glycerol, and LB with 0.12% MgSO₄, respectively. TB medium exhibited more than two and half (about 2.5) fold increase in the recombinant Sap expression as compared to LB medium (Table 1).

Generally, LB medium is commonly used for small-scale production of recombinant proteins. However, for production of recombinant proteins in fermenter, it is important to optimize different growth media. In this study, TB growth medium resulted in highest biomass and enhanced protein expression in comparison to other growth media used. The higher biomass resulted in enhanced protein production. Moreover, glycerol present in the growth medium might have contributed towards protein folding and solubility.

Scale up of recombinant surface array protein using bioreactor

Scale up of recombinant Sap was done by batch cultivations (5 L bioreactor). The final wet cell weight was 10.4 g/l after batch fermentation process. The recombinant Sap yield at shake flasks and batch fermentation process has been given in Table 1.

It is desirable to produce the recombinant proteins in higher amounts for use in detection/diagnostic assays. Therefore, it is important to devise the scale up process of such proteins. Several proteins with diagnostics, prophylactics or therapeutics applications have been efficiently produced by batch fermentation (Tripathi 2016). In this report, the final wet biomass of batch fermentation process was two- and three times higher than that in shake flasks using LB and TB growth media, respectively.

Purification of recombinant Sap

The biomass recovered from shake flask cultures and batch fermentation processes was processed for purification of protein. The pellet was lysed and purified using immobilized metal affinity chromatography with 5 ml pre-packed column on Akta Explorer FPLC system (Fig. 1). The purity of eluted proteins was found to be more than 95% upon SDS-PAGE analysis (Fig. 1). The affinity purified recombinant protein was diafiltered and used for generation of antibodies. A protein yield of 46 and 20 mg/l of culture was obtained during batch process and shake flask culture, respectively (Table 1). Batch process yielded more than twofold recombinant Sap in comparison to shake flask culture (Table 1).

Table 1 Comparison of recombinant surface array protein expression levels in recombinant *E. coli* grown using different culture medium and cultivation conditions

Media type	Cultivation mode	Wet cell weight (g/l)	Specific product yield (mg/g)	Final product concentration (mg/l)
LB	Shake flask	3.0	2.36	7.1
TB	Shake flask	5.1	3.92	20
TB	Batch fermentation	10.4	4.03	46

Fig. 1 Chromatogram of affinity purification of recombinant Sap. Inset: Purification and characterization of recombinant Sap. (a): SDS PAGE. Lane 1, MW marker; lane 2, flow through; lane 3, wash fraction; lane 4–9 elutes

Evaluation of antibody titre using Indirect ELISA

Purified recombinant Sap was used to raise polyclonal antibodies in rabbit. The antibody titre of rabbit serum was found to be 1:2,56,000. Pre-immunization serum was used as control in ELISA.

Evaluation of recombinant Sap as an antigen for detection of *B. anthracis*

To evaluate the detection potential of antibodies raised against purified Sap, Western blot was used. The culture supernatant of *B. anthracis* Sterne was collected hourly to check the release of Sap in broth. Sap could be detected after 6 h of bacterial growth in BHI by Western blot using rabbit anti-Sap antibodies (Fig. 2). Among the 6 different *Bacillus* species tested, only *B. anthracis* Sterne was found positive by Western blot, and all the other species were found negative for secretion of Sap after 16 h of growth by Western blot. Thus, the results revealed that Sap is the specific marker which is released by *B. anthracis* just after 6 h of growth. Hence, Sap can be an important biomarker for detection of *B. anthracis*. Thus, this study proved that purified Sap harnesses great potential towards development of specific, rapid and field deployable immunological systems for detection of *B. anthracis* using antibodies raised against Sap in different animals.

There is a need for rapid and reliable detection system for *B. anthracis*, which is an important bioterror agent. Traditionally, the presence of *B. anthracis* is established by gram staining or capsule staining in culture (Caksen et al. 2001). There are several serodiagnostic assays for anthrax

Fig. 2 Detection of Sap in *B. anthracis* Sterne culture supernatant in Western blot. Lane 1, MW marker; Lane 2, 1 h grown culture; Lane 3, 2 h grown culture; Lane 4, 3 h grown culture; Lane 5, 4 h grown culture; Lane 6, 5 h grown culture; Lane 7, 6 h grown culture; Lane 8, 7 h grown culture; Lane 9, 8 h grown culture; Lane 10, Un-inoculated broth

infection in humans (Ghosh and Goel 2012; Ghosh et al. 2013b, c, 2015). However, detection of *B. anthracis* directly from environmental or clinical samples requires sophisticated techniques (Ghosh et al. 2013a; Goel et al. 2005; Pal et al. 2016). Therefore, it is important to develop a simple and reliable detection method for *B. anthracis*. Sap is a potential biomarker for detection of *B. anthracis*. In this study, the ability of the recombinant Sap was evaluated for detection of *B. anthracis* by Western blot. *Bacillus anthracis* culture supernatant specifically tested positive in Western blot using anti-Sap antibodies (Fig. 2). Further, all the other *Bacillus* species including *B. cereus*, *B. globigii*, *B. pumilus*,

B. subtilis and *B. thuringiensis* were found negative for Sap in Western blot, which ascertained the specificity of this assay. Thus, Sap can be an important detection biomarker for *B. anthracis*. However, further efforts are required to develop the specific antibodies using Sap for development of rapid immunological detection systems like ELISA and immuno-chromatography tests (ICTs).

In this study, for the first time, we report the enhanced production of recombinant Sap in *E. coli* in soluble form at shake flask level with a yield of 20 mg/l, and easily scalable to 46 mg/l in a bioreactor. The successful production of Sap coupled with one-step purification procedure will certainly be useful for development of detection systems for *B. anthracis*. Such a strategy of expressing recombinant surface array protein in soluble form demonstrates the prospect of high yield production of diagnostic proteins, and the same could be applied to other proteins also.

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Compliance with ethical standards

Conflict of interest We have no conflict of interest.

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