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An efficient fusion protein system for expression of *Bacillus anthracis* protective antigen as immunogenic and diagnostic antigen

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ABSTRACT

Objective: To produce high quantities of recombinant protective antigen (rPA) for human vaccine and diagnosis. **Methods:** The PA gene was amplified by PCR with pXO1 plasmid as template. The PCR product was cloned into pMAL-c2X vector using the *BamH*I and *Sal*I restriction enzymes. The recombinant plasmid was transformed into *Escherichia coli* DH5 α strain and then screened for transformation. The expression of protective antigen was analyzed by SDS-PAGE and Western blotting after isopropyl β-D-thiogalactopyranoside (IPTG) induction. **Results:** The full-length PA gene (2.2 kb) was cloned into pMAL vector system. The recombinant vector was confirmed by restriction enzyme and PCR analysis. The expression of cytoplasmic maltose-binding protein-protective (MBP-P) antigen fusion protein was detected by SDS-PAGE and Western blotting, and obtained a 125 kDa protein band, which was similar to expected size of fusion protein. **Conclusions:** This expression system can be used in the high production of rPA. After purification and immunization studies, the purified rPA may be used in the development of the human recombinant anthrax vaccine and also in diagnosis of anthrax disease.

1. Introduction

Anthrax, a fatal bacterial disease, is caused by the gram-positive, spore-forming bacterium, *Bacillus anthracis (B. anthracis)*[1, 2]. The disease normally occurs in herbivores, and human infections usually occur after contacting with infected animals or their contaminated products[3]. Also, anthrax may be used as a biological weapon[4]. The two principal virulence factors of *B. anthracis* are a poly- γ-D-glutamic acid capsule and the anthrax toxin. These factors are encoded by virulence plasmids pXO2 and pXO1, respectively[5]. Anthrax toxin consists of three components: protective antigen (PA, 83 kDa), edema factor (EF, 89 kDa), and lethal factor (LF, 85 kDa). EF is a calcium and calmodulin-dependent adenylate cyclase that increases intracellular cAMP levels. LF is a zinc metalloprotease that cleaves the N-terminal of mitogen-activated protein kinase kinases (MAPKKs). PA83 binds to a cell receptor and then is cleaved by furin-like proteases into PA20 and PA63 fragments. PA63 forms a ring-shaped heptamer structure at the cell surface and interacts with EF or LF. This complex is internalized by receptor-mediated endocytosis, where at low pH of the endosome, PA heptamer forms a pore that inserts EF and LF into the cytoplasm of the target cell[6-9]. Individually, these components are not toxic, but the combinations of PA with EF or LF produce edema toxin or lethal toxin, respectively[10-12]. The lethal toxin causes death in animal models and the edema toxin causes local edema at the inoculation site[13].With respect to the acuteness of this disease, immunization before challenge is of crucial importance. The current licensed human vaccines are produced in the United States and United Kingdom. The US vaccine (AVA) is an aluminum hydroxide-adsorbed cell-free culture filtrate of a non-encapsulated strain (V770-NP1-R), and the UK vaccine is an alum-precipitated cell-free culture filtrate of a Sterne strain (34F2)[14,15]. These vaccines can cause local side effects, and several boosters are required to maintain protective immunity. Therefore, an improved, effective, and safe human vaccine is needed against anthrax. PA is the major immunogenic component in the current licensed human vaccines[16,17]. Thus, purified recombinant PA can be used for development of a human subunit vaccine against anthrax. Researchers have cloned and expressed the PA gene in several bacterial hosts. In the

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present study, we developed a vector system for high level expression of rPA in *Escherichia coli(E. coli)* DH5 α host strain.

2. Materials and methods

2.1. Reagents and chemicals

T4 ligase, restriction enzymes, isopropyl- β -D-thiogalactopyranoside (IPTG), 1kb DNA ladder, and protein molecular weight marker were obtained from Fermentas (USA). Plasmid isolation kit and gel extraction kit were from Qiagen (Germany). Taq polymerase and dNTPs were from Cinnagen (Iran). Oligonucleotide primers were synthesized by TAG Copenhagen (Denmark). Secondary antibody and nitrocellulose membranes were from Komabiotech (Korea). Anti-PA monoclonal antibody was from Hytest (Finland). All chemicals were purchased from Merck (Germany), Sigma (USA), and Applichem (Germany).

2.2. Bacteria, plasmid and culture media

B. anthracis Sterne strain 34F2 was obtained from anthrax live spore vaccine (RAZI vaccine and serum research institute, Iran). *E. coli* DH5 α host strain was obtained from Cinnagen. The pMAL-c2X expression vector was purchased from New England Biolabs (USA). *E. coli* DH5 α was grown in LB broth or LB agar (Merck) and *B. anthracis* Sterne strain was grown in brain-heart infusion (BHI) medium (Difco, USA). When required, LB medium was supplemented with 100 μg/mL ampicillin.

2.3. Bacterial growth and isolation of plasmid DNA

B. anthracis was grown overnight in BHI medium at 37 ℃, 180 rpm. Isolation of pXO1 plasmid was carried out by alkaline lysis method[18].

2.4. Polymerase chain reaction (PCR)

The coding region of PA (2.2 kb) was amplified by PCR using the oligonucleotide primers 5′- G C C G G G A T C C G A A G T T A A A C A G G A G A A C-3′, containing *BamH*I restriction site (underlined) and 5′-G C C G G T C G A C T A G A A T T A C C T T A T C C-3′, containing *Sal*I restriction site (underlined) with pXO1 as a template. PCR was performed in a Eppendorf thermal cycler (Germany) and in a total volume of 50 μL(1.5 mM $MgCl_2$, 1 pmol of each primers, 0.2 mM dNTPs, and 2.5 unit Taq polymerase). Denaturation, annealing, and extension were carried out at 94 ℃ for 60 s, 49 ℃ for 60 s, and 72 ℃ for 90 s, for 35 cycles. The PCR product was analyzed by 1% agarose gel electrophoresis.

2.5. Cloning of PA gene into E. coli DH5 α

pMAL-c2X plasmid was isolated and purified by plasmid isolation kit. The PCR product was purified from the agarose gel by gel extraction kit. The purified PCR product and pMAL-c2X vector were digested with *BamH*I and *Sal*I restriction enzymes. The digested products were analyzed on 1% agarose gel and purified by gel extraction kit. The digested PCR product was ligated into pMAL-c2X vector using T4 ligase and incubated at 4 ℃ overnight. The pMAL-c2X-PA vector was transformed into *E. coli* DH5 α host strain by one-step PEG transformation method[18]. The colonies on LB agar supplemented with ampicillin were screened by PCR analysis using PA gene specific primers. Plasmid DNA was miniprepared from positive clones and isolation of PA gene fragment was analyzed on 1% agarose gel by restriction enzyme digestion.

2.6. Expression of the recombinant fusion protein

A positive clone was selected and grown overnight in 5 mL LB broth supplemented with ampicillin at 37 ℃, 180 rpm. 100 μL of this culture was inoculated to 10 mL of LB broth supplemented with glucose and ampicillin. Bacterial culture was grown at 37 ℃, 180 rpm and induced with 0.3 mM IPTG at OD_{600} equal to 0.5. The culture samples were collected at 2, 4 and 6 h after IPTG induction. The cells were harvested by centrifugation at 13 000 g for 2 min and the pellet resuspended in sample buffer. Samples were boiled in sample buffer for 5 min and centrifuged at 13 000 g for 1 min. The supernatant of samples was analyzed by SDS-PAGE on 10% polyacrylamide gel followed by Coomassie Brilliant Blue staining. The recombinant MBP-PA fusion protein was also detected by Western blotting using an anti-PA monoclonal antibody. After SDS-PAGE of samples, proteins were transferred to nitrocellulose membrane by electrophoresis at 60 V for 4 h using transfer buffer. The membrane was blocked with 5% skim milk in PBS overnight. After washing with PBST (PBS containing 0.05% Tween 20) for three times, each for 5 min, the membrane was incubated with 1:10 000 dilution of anti-PA monoclonal antibody in the PBST containing 2% skim milk for 1 h. The membrane was washed with PBST as described above and incubated with 1:1 000 dilution of goat anti-mouse horseradish peroxidase-conjugated polyclonal antibody in the PBST containing 2% skim milk for 1 h. Finally, the membrane was washed with PBST for three times, each for 10 min, and HRP-conjugated secondary antibody was detected using 4-chloro-1-naphthol and H_2O_2.

3. Results

3.1. Construction of recombinant plasmid

For expression of the *B. anthracis* protective antigen protein in *E. coli*, the PA gene was amplified by PCR using specific primers containing *BamH*I and *Sal*I restriction sites and pXO1 plasmid as template. The PCR product was confirmed on agarose gel and a DNA fragment with expected size (2.2 kb) was observed. After PCR amplification, the PA gene was ligated into pMAL-c2X vector using *BamH*I and *Sal*I restriction sites. In this recombinant construct, the PA gene was fused to *malE* gene that encodes the maltose-

binding protein (MBP) and both genes were under control of P_{tac} promoter.

3.2. Transformation and clone screening

The pMAL-c2X expression vector was transformed into *E. coli* DH5 α using one-step PEG method. The colonies were screened by PCR and positive clones were confirmed by presence of a 2.2 kb DNA fragment on agarose gel(Figure 1). The purified recombinant plasmids of positive clones were digested with *BamH*I and *Sal*I, and the insert was released from plasmids that corresponded to the PA gene size (Figure 2).

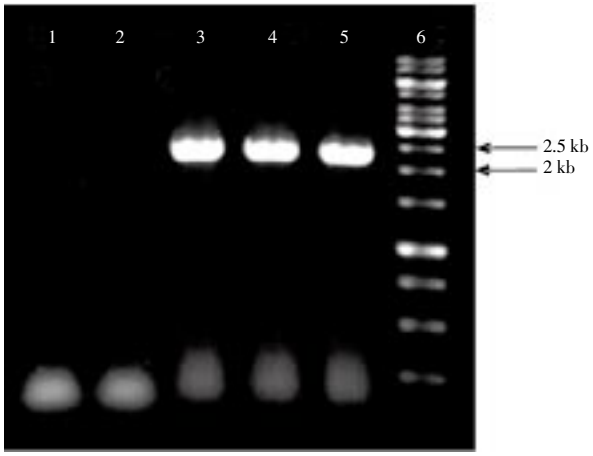

Figure 1. Screening of the clones by PCR amplification.
Lane 1: negative control, lane 2: negative clone, lanes 3, 4 and 5: PCR product of positive clones, lane 6: 1kb DNA ladder.

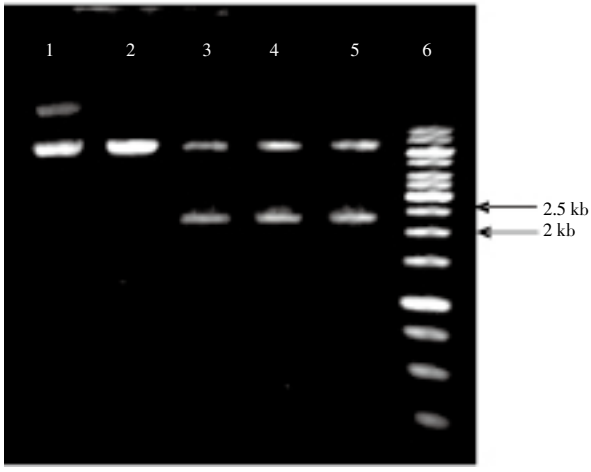

Figure 2. Restriction digestion analysis of positive clones.
Lane 1: undigested pMAL-c2X, lane 2: pMAL-c2X digested by *BamH*I, lanes 3, 4 and 5: pMAL-c2X-PA digested by *BamH*I and *Sal*I, lane 6: 1 kb DNA ladder.

3.3. Expression of rMBP-PA fusion protein

After Coomassie Blue staining, a protein band with expected molecular weight of about 125 kDa was obtained, when compared to the uninduced and control samples(Figure 3). The expression of rMBP-PA fusion protein was also confirmed by Western blot analysis using anti-PA monoclonal antibody (Figure 4). Western blotting detected a band at the expected position and the rPA was recognized by anti-PA antibody. In the uninduced and control samples, no reaction was observed.

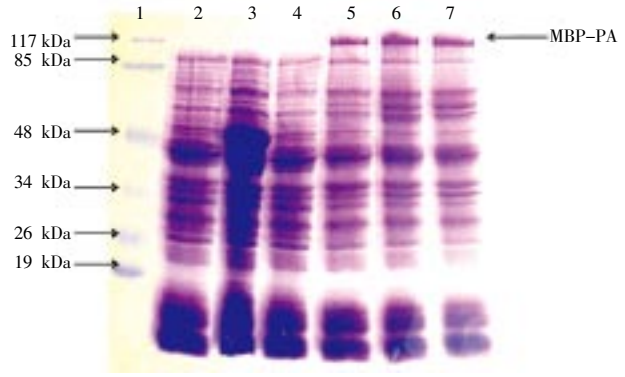

Figure 3. SDS-PAGE analysis of the MBP-PA fusion protein expression in *E. coli* DH5 α host.
Lane 1: protein molecular weight marker, lane 2: negative clone (pMAL-c2X) before IPTG induction, lane 3: negative clone after induction, lane 4: positive clone (pMAL-c2X-PA) before induction, lanes 5, 6 and 7: positive clone 2, 4 and 6 h after induction.

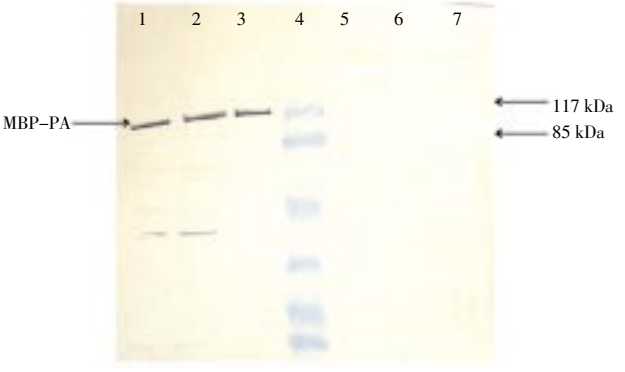

Figure 4. Western blot analysis of the MBP-PA fusion protein expression.
Lane 1, 2 and 3: positive clone (pMAL-c2X-PA) 6, 4 and 2 h after induction, lane 4: protein molecular weight marker, lane 5: positive clone before induction, lane 6: negative clone (pMAL-c2X) before induction, lane 7: nagative clone after induction.

4. Discussion

Anthrax is a serious problem in developing countries and occurs among animals and humans. The current threat of anthrax is potential use as a biological weapon and *B. anthracis* is an attractive agent for this purpose[13]. Thus, vaccination of humans against anthrax is essential. The current available PA-based vaccines have several disadvantages and are not ideal for human immunization[17]. For these reasons, a new vaccine should be improved, safe, cheap, and effective against all forms of anthrax infection (cutaneous, inhalational, and gastrointestinal). One of the toxin components is PA, which translocates LF and EF into the cytoplasm of the target cell[7,8]. PA is an essential component for designing an effective anthrax vaccine and anti-PA antibodies induce protection against

disease. Antibodies against PA could inhibit the toxin function and germination of spores[6, 19]. Therefore, the PA gene has been expressed in several expression systems including *E. coli*[20,21], *Bacillus subtilis (B. subtilis)*[22], *Bacillus brevis (B. brevis)*[23], *Baculovirus*, and *Vaccinia* virus[24]. Recently, the most production of PA has been reported from *E. coli*, approximately 370 mg/mL of culture[21]. In the present study, we used the pMAL expression system for high expression of PA in *E. coli*. The results showed that the PA gene was cloned successfully downstream to the *malE* gene of *E. coli* and expressed as a fusion protein with MBP of *E. coli*. The expected MBP-PA fusion protein was expressed with a molecular weight of about 125 kDa and observed in SDS-PAGE analysis. The fusion protein identity was also confirmed by Western blotting using monoclonal antibody. The pMAL expression and purification system has the following advantages: 1) protein expression is controlled by the strong tac promoter and induced in the presence of IPTG, 2) the recombinant fusion protein is purified by one-step purification using affinity for maltose that facilitate purification process of foreign protein and 3) the pMAL-c2X vector encodes a site for factor Xa, which cleaves MBP from the fusion protein after purification. In most cases, the pMAL-c2X plasmid expresses fusion protein, which approximately constitutes 20%-40% of the total cellular protein. In the present study, Western blot analysis indicated that proteolysis of induced fusion protein increased with IPTG induction time.

In conclusion, we successfully expressed *B. anthracis* PA (83 kDa) at high level in the *E. coli* DH5 α strain. Further studies including purification and immunization in animal models will be done to evaluate potential of the purified PA as a recombinant vaccine. This antigen could also employ in anthrax diagnostic tests and laboratory research.

Conflict of interest statement

We declare that we have no conflict of interest.

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