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Immobilization of alpha toxin on the surface of the *bacillus subtilis* spores

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ABSTRACT

All strains of *Clostridium Perfringens* have gene encoding alpha toxin. Alpha toxin is important in pathogenesis of several diseases from gas gangrene to necrotic enteritis in chickens. The toxin is consists of two domains, the N-domain containing the site for phospholipid hydrolysis and the C-domain plays a role in haemolytic and sphingomyelinase activities. The presence of the C-domain is crucial for recognition of the cell membrane phospholipids by the toxin. It is shown that Immunization against the C-terminal domain of the toxin can make protection, at least partially against diseases like gas gangrene.

On the other hand, *Bacillus subtilis* spores are known to be GRAS, highly resistant to harsh conditions and inducing protective immune response. These attributes make *Bacillus subtilis* spores an ideal candidate for drug and vaccine delivery.

At the present study, as a preliminary step toward developing an effective vaccine against necrotic enteritis, the immunogenic region of alpha toxin is cloned, expressed and purified. The purified region was then covalently immobilized on the surface of the *Bacillus subtilis* spores, using non-genetically approach. The presence of alpha toxin on the spore surface is then verified by western blotting. In conclusion, C-terminal fragment of alpha toxin was successfully displayed on the spores of *Bacillus subtilis* using a non-genetically approach.

Keywords: alpha toxin, *bacillus subtilis,* Immobilization.

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Introduction

A wide range of experiments shows the role of alpha toxin from *Clostridium perfringens* (CP) in the pathogenesis of many diseases ranging from gas gangrene to several gastrointestinal diseases in human and animals (1-5). As the main toxin produced by all types of CP, alpha toxin shows platelet aggregation, hemolysis effects on cells, contraction of blood vessels and phospholipase activity (6-8).

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Immunization of mice with alpha-toxoid can protect them from gas gangrene (9). Vaccination of calves with the C-terminal domain of alpha toxin can partially protect them against bovine necrohemorrhagic enteritis (4). It is also shown that this toxin play an important role in pathogenesis of avian necrotic enteritis (NE) (10-12). NE costs more than 2 billion dollar a year for poultry industry worldwide (13). Furthermore it is claimed that alpha toxin is associated with some diseases like Crohn's disease (14). Some researchers also showed that sudden infant death syndrome (SIDS) is associated with alpha toxin in some cases (15, 16).

Alpha toxin consists of two domains; an N-terminal domain and a C-terminal domain (2). The former has phospholipase C activity on phosphatidylcholine (17) and the latter is essential in binding of the toxin to the membrane (6).

Substitution of Tyr-275, 307 and 331 residues by Asn, Phe, or Leu causes significant reduction in binding of the toxin to the membrane (18). Although the N-terminal domain of alpha toxin is enzymatically active in the absence of the C-domain, it has no hemolytic activity and toxic effects (17, 19). This confirm that binding of the alpha toxin to the target cell membrane is crucial for its cytotoxicity. This attachment is shown to be mediated through GPI-anchored proteins as the receptor (20).

On the other hand, displaying of recombinant proteins on the surface of bacterial cells and spores has drawn a lot of attention in biotechnology as a vaccine candidate (21, 22). In this context, special attention is given to the *Bacillus subtilis* spores. A number of reasons support the use of *Bacillus subtilis* spores as a suitable vaccine delivery vehicles. *Bacillus subtilis* as well as its spores are known to be GRAS (Generally recognized as safe). *Bacillus* spores are highly resistant to the different harsh environmental conditions (23), can act as adjuvant (23) and can be used for delivery of heterologous proteins and vaccine candidates to the gastrointestinal tract (24).

In the present study, the immunogenic epitopes of alpha toxin was determined using bioinformatics tools. Then the selected part of the toxin was cloned, expressed in *E. coli* and purified. Finally, alpha toxin was attached to the surface of *Bacillus subtilis* spores non-genetically and surface immobilization was evaluated by western blotting.

Materials and Methods

Sequence analysis

Amino acid sequence of "Alpha toxin" (Acc. No. CAA35186.1) was obtained from GenBank. Antigenic properties was determined by submitting the toxin sequence into VAXIJEN software (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). The potential epitopic regions of the structure of the protein were predicted using BCEPRED online tool (http://www.imtech.res.in/raghava/bcepred/bcepred_submission.html).

Cloning and protein expression

The selected part of the gene was synthesized at ShineGene Molecular Biotech, Inc (Shanghai, china). pUC57 plasmid harboring the recombinant gene was transformed into the competent *E. coli* DH5α for amplification. The presence of the recombinant plasmid in the bacteria was confirmed by ampicillin selection and other control tests. The extracted pUC57 and pET-26b(+) plasmids were digested by NdeI and XhoI restriction enzymes. The approximately 351 bp

fragment was purified and inserted into the pET-26b (+) expression vector. Then plasmid was transformed into competent host *E. coli* BL21(DE3). Transformed bacteria were cultured in LB medium at 37 °C. After the OD_{600} of bacterial culture reached 0.6-0.7, the expression of recombinant protein was induced by isopropylthio-β- D-galactoside (IPTG) at a final concentration of 1 mmol/L and then incubated for 5 hour at 30 °C. Finally, bacterial pellet was collected by centrifugation.

Protein purification

His-tagged recombinant protein was purified by Ni-NTA Magnetic Agarose Beads (QIAGEN Inc, USA). The bacterial pellet was suspended in lysis buffer (8 M Urea, 0.1 M NaH2PO4, 0.01 M Tris·Cl, 0.05% Tween 20, pH 8.0) and was shaken at room temperature for 1 Hour. The cell lysate was then centrifuged at 10000g for 30 min and the cleared supernatant was added to Ni-NTA resins. The suspension of resins and the cell lysate was incubated for 2 hour at room temperature, resins were washed 5 times with washing buffer (8 M Urea, 0.1 M NaH2PO4, 0.01 M Tris·Cl, 0.05%, imidazole 20 mM). At last, the recombinant protein was eluted by adding elution buffer containing (8M Urea, 0.1 M NaH2PO4, 0.01 M Tris·Cl, 0.05%, imidazole 250 mM). Protein samples were analyzed by SDS-PAGE analysis and western blotting.

Spore preparation and purification

Sporulation of *Bacillus subtilis* 168 *trpC2* was induced by the exhaustion method in Difco-Sporulation Medium (DSM). After sporulation, media containing spores were centrifuged at 10,000g, 20 min, 4°C. The pellet was treated with a solution containing MgSO 4 (2.5 µg/mL), lysozyme (200 µg/mL) and DNase I (2 µg/mL) for 30 min at 37 °C. In order to inactivate enzymes and ensure the killing of vegetative cells, spores were then heat-treated at 68 °C for (45 min). After repeated washing steps with distilled water, purified spores were stored at -20 °C until use.

Immobilization on the surface of the spores

Covalent immobilization of the recombinant protein onto the *Bacillus* spore surface was performed as described previously (25). Briefly, functional groups of the spore surface was activated using glutaraldehyde. The solution of alpha toxin (100 $\mu g.ml^{-1}$) in 50 mM phosphate buffer (pH 7.5) was then added to the spores suspension at room temperature for 2 hour. Spores were harvested by centrifugation (4000 g, 10 min) and washed three times with 50 mM phosphate buffer (pH 7.5). A high concentration of salt solution (1 M NaCl) was used for washing of the suspension to eliminate non-covalently adsorbed recombinant proteins on the spore surface.

Spore decoating

Spore surface proteins including the recombinant protein were extracted by incubation of the manipulated spores in decoating extraction buffer (50 mM Tris–HCl (pH 8.0), 1 % (w/v) sodium dodecyl sulfate (SDS), 8 M urea, 50 mM dithiothreitol, 10 mM EDTA) for 90 min at 37 °C (26). The spore suspension was then centrifuged and the supernatant containing the recombinant protein was analyzed by SDS-PAGE and Western blotting to confirm the attachment of the recombinant protein onto to the spore surface.

SDS-PAGE and Western blot analysis

Extracted proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE). Western blotting against the recombinant protein was carried out using a monoclonal anti-his tag antibody (Roche, USA). The recombinant protein

was separated on SDS-polyacrylamide gels and transferred to PVDF membrane (Roche, Germany). Further analysis was done according to the manufacturer instruction.

Results

Gene synthesis

A C-terminal region (amino acids number 256-370) of *Clostridium perfringens's* alpha toxin was selected for construction of gene expression cassette.

Prediction of mRNA structure

Folding of the mRNA for alpha toxin was predicted by mfold online software (http://unafold.rna.albany.edu/). As it is shown in figure 1, no long stable hairpin pseudoknot is observed at the initializing 5' nucleotides of the mRNA. The minimum free energy of the secondary structure formed by RNA was also predicted as -86.8 kcal/mol.

B-cell epitopes

The sequence of the alpha toxin was submitted to VaxiGen online software while the cutoff value was set to 0.4 (Table 1). The selected region of the alpha toxin was predicted to be immunogenic. The epitopic regions were then mapped by BCEPRED online service for alpha toxin.

Table 1. Scores of alpha toxin for being antigen

Toxin	Ag Score
Alpha Toxin	0.52
Alpha toxin (amino acids 256-370)	0.72

Protein expression and purification

E. coli BL21DE3 strain transformed with plasmid pET-AT was induced by IPTG at OD_{600} 0.6. The recombinant Alpha toxin was extracted and purified by nickel beads according to the

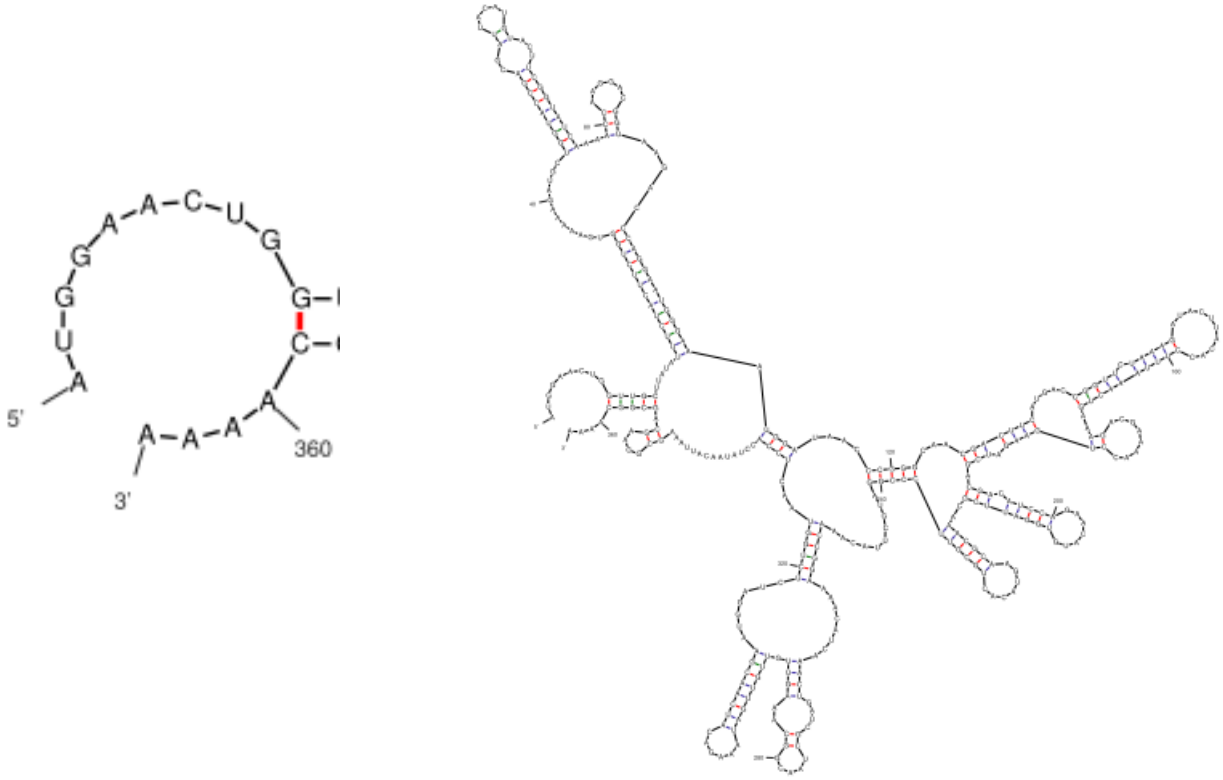

Figure 1. mRNA stability analysis by mfold software.

manufacturer instruction (QIAGEN inc, USA, 2001). SDS PAGE 12% revealed a single band between molecular weight markers of 18.4 and 14.4 kDa were corresponds to the alpha toxin with the molecular weight of 15 kDa (Figure 2).

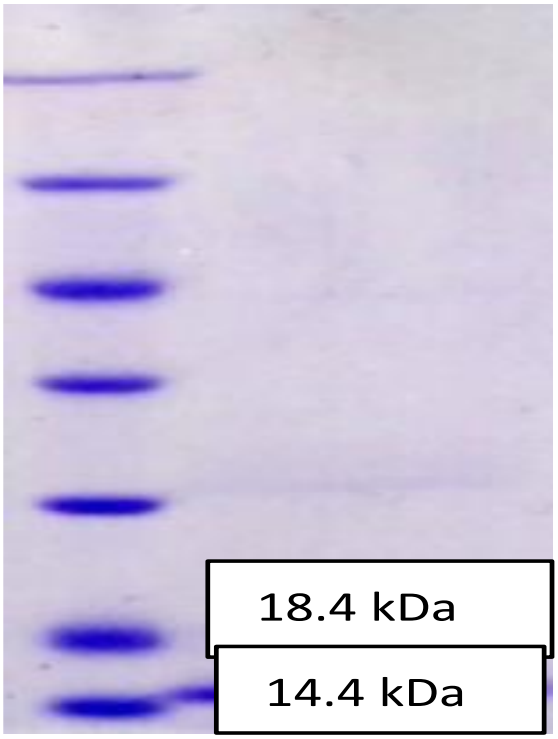

Figure 2. Visualization of the alpha toxin on SDS-PAGE.

Western blotting

To verify the attachment of recombinant alpha toxin on the spore surface, after treatment of the free and modified spores with decoating buffer, surface proteins were extracted from spore surface and were analyzed by SDS-PAGE and western blot analysis.

As it is shown in figure 3, western blot analysis using a monoclonal anti-his tag antibody (Roche, USA) showed a single band representing alpha toxin with approximate molecular weight of about 15 KDa was detected which is absent in the free spore surface extracted proteins.

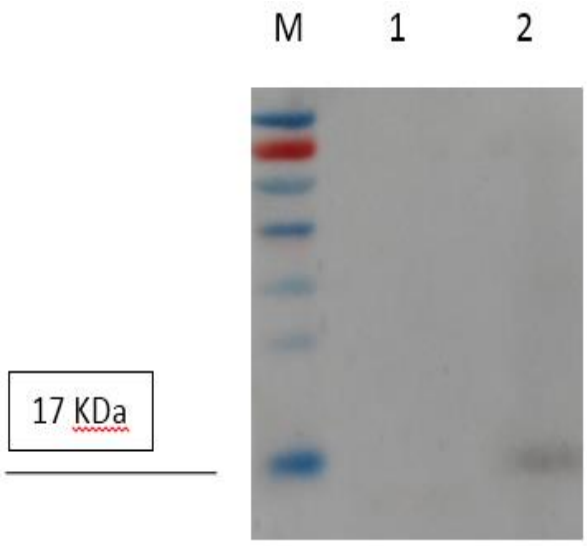

Figure 3. Purification of the alpha toxin by Ni-NTA and further analyzing by western blotting. M: protein weight molecular marker, lane 1: un-induced total lysate of the *E. coli* bl21 (DE3), lane 2: western blotting of the purified alpha toxin.

Discussion

Alpha toxin plays an important role in pathogenesis of many diseases associated with *C. perferingens.* These include gas gangrene, necrotic enteritis, bovine necrohemorrhagic enteritis and Crohn's disease (4, 9, 10). It is shown that vaccination against C-terminal fragment of alpha toxin is effective against *C. perfringens* pathogenesis (3, 27).

The present study demonstrates the successful non-genetically immobilization of C-terminal domain of alpha toxin from *C. perfringens* on the surface of *Bacillus* spores. *Bacillus subtilis* is classified as a GRAS organism (generally recognized as safe). Bacillus spores benefit from some advantages including resistance to extreme temperatures, lytic enzymes and toxic chemicals, low cost maintenance and work as adjuvants. These features make spores an interesting platform

for delivery of drugs and vaccine candidates (28, 29).

As it is shown in Figure 1, the 5' terminus folding of mRNA is similar to the other bacterial gene folding structure. No long stable hairpin pseudoknot is observed at the initializing 5' nucleotides of the mRNA. The C-terminal domain of alpha toxin is seemed to be the antigenic part of the whole toxin as is predicted by bioinformatics tools (table 1). His-tagged truncated alpha toxin was successfully expressed in *E. coli* and purified using nickel beads (Figure 2). The purified alpha toxin is then immobilized on the surface of *Bacillus* spores using a glutaraldehyde. Washing of spores with high concentration of salt ensures that the attachment is carried out through just covalent immobilization on the surface of the spores. As indicated in Figure 3, western blot analysis of extracted spores surface proteins using a monoclonal anti-His antibody conjugated with HRP antibody confirm the presence of alpha toxin on the surface of the spores.

In recent years, many concerns raised globally toward using of GMO (Genetically Modified Organisms). As indicated here, the recombinant alpha toxin was attached onto the surface of spores as a live, safe carriers in a non-genetically approach. This approach for immobilization of drugs and vaccine candidates on the surface of spores can be used as a good alternative for DNA manipulated bacteria and spores surface display systems.

Conclusion

In conclusion, this is the first report describing covalent immobilization of alpha toxin on the surface of *Bacillus subtilis* spores using a non-genetically approach.

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Conflict of Interest

The authors declare no conflict of interest.

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