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Full Length Article

Expression of *Clostridium perfringens* Alpha-Epsilon Soluble Fusion Toxin Gene in *Escherichia coli* and its Immunogenicity in Mice

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Abstract

In this study, a set of molecular biotechnologies were used to efficiently express and obtain soluble fusion protein of alpha-epsilon-toxin (α-ε-toxin) derived from *Clostridium perfringens* in *Escherichia coli*. We have successfully obtained a soluble fusion protein of α-ε-toxin for the first time with efficient expression in *E. coli* system by conducting codon optimization, removing the signal peptide, selecting sequences of higher hydrophilicity and antigenicity, removing the lethal gene and optimizing the expression conditions. Noticeable rise of antibody level was detected in the serum of mice after immunization with the expressed protein. The immunized mice got protected against *C. perfringens* type A, B, C and D with the survival rate of 100, 90, 85 and 100%, respectively.

Keywords: *Clostridium perfringens*; Alpha-epsilon toxin gene; Immunogenicity; Soluble expression and purification; Soluble fusion protein

Introduction

Clostridium perfringens is an important human and veterinary pathogen. It is highly associated with the diseases including braxy, lamb dysentery, hemorrhagic enteritis in cattle and sheep and ovine/bovine enterotoxemia, which cause great economic loss in livestock husbandry system (Ewoldt and Anderson, 2005; Songer and Miskimins, 2005; Kalender *et al.*, 2009; Lebrun *et al.*, 2010). In the meanwhile, *C. perfringens* is one of the major pathogens associated with the human diseases such as food poisoning and traumatic gas gangrene (Welch and Nuttall, 1892; Lucey and Hutchins, 2004; Morris, 2009; Sakurai *et al.*, 2009; Savic *et al.*, 2012). Alpha and epsilon toxins are the major exotoxins secreted by *C. perfringens*. However, the most of the reported expressions of the alpha and epsilon proteins are inclusion bodies. Moreover, the expressed alpha and epsilon proteins only take very low percentage of the total amount of the bacterial proteins. The expression and purification methods are relatively complex. An expression of soluble proteins with higher immunogenicity is still absent (Chandran *et al.*, 2010; Langroudi *et al.*, 2011; Zeng *et al.*, 2011; Pilehchian *et al.*, 2013). Therefore, *in vitro* expression of soluble fusion alpha-epsilon-exotoxin proteins without destroying its immunogenicity is very important for the function study of *C. perfringens* exotoxins as well as the development of related genetically engineered subunit vaccines. As such, for inhibition and control of *C. perfringens*-associated diseases, there is an urgent need to develop soluble fusion genetically engineered multivalent subunit vaccines or nucleic-acid vaccines to provide protection against infection by multiple types of *C. perfringens* bacteria.

Materials and Methods

Animals

A total of two hundred 56-day-old female BALB/C mice (18–22 g in weight) were randomly assigned into two groups: one group with 160 mice was divided into 4 subgroups with 40 mice of each, which were immunized respectively with the expressed proteins followed by challenging with the toxin; the other group, served as control, was divided into 4 subgroups with 10 mice of each, which were immunized with PBS and underwent same challenging.

Vectors: the pET32a expression vectors were purchased from Novagen Inc., Madison, WI, USA.

Competent cells: BL21(DE3) competent cells were purchased from Beijing TransGen Biotech Co., Ltd., Beijing, China.

Enzymes and reagents: restriction enzymes *Bam*HI and *Xho*I, 2000 DNA Marker, T4 DNA ligase, SDS, IPTG and

Taq PCR Master Mix were provided by Takara Biotechnology Co., Ltd., Dalian, China. Agarose, DNA Rapid Purification and Recovery Kit, DNA Extraction Kit and Plasmid Rapid Isolation Kit were all purchased from Beijing TransGen Biotech Co., Ltd., Beijing, China. The pre-stained protein maker was purchased from Beijing TransGen Biotech Co., Ltd., Beijing, China. The His-tag protein purification kit (10 mL) and the size exclusion chromatography columns (Superdex^{TM} 2000) were purchased from GE Healthcare Bio-Sciences, Pittsburgh, P.A., U.S.A. The horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and Freund's complete and incomplete adjuvants were purchased from Sigma-Aldrich Co. LLC., Beijing, China. Bacterial strains: *C. perfringens* virulent strains, *i.e*., type A (C57-10), type B (C58-5), type C (C59-4) and type D (C60-11), were produced by China Institute of Veterinary Drug Control.

Gene Synthesis

Sequencing and synthesizing of the fusion protein target gene was completed by BGI, Shenzhen, China.

Synthesis of the Alpha-epsilon-toxin Fusion Gene

Toxin alpha (amino acids Trp^{29} to Lys^{258}, GenBank Accession No: ABA55036.1) was fused to toxin epsilon (amino acids Lys^{33} to Lys^{328}, GenBank Accession No: Q02307.1). The signal peptide of the fusion protein was removed and the codon was optimized. The genes α-ε was obtained by chemical synthesis.

Construction and Transformation of Recombinant Expression Vectors

The synthetic α-ε gene was used as the template and was amplified by overlapping polymerase chain reaction (PCR) using the forward primer F1 (5'-*ggatcc*atgtgggatggaaaaattgatgga-3') and the reverse primer R1 ((5'- *ctcgag*tcatttgatgcccggtgctttga-3'). The target plasmid was subsequently transformed into *Escherichia coli* (*E. coli*) competent cells BL21(DE3). Cultures of the *E. coli* strain BL21(DE3) were uniformly smeared on L broth (LB) plates (supplemented with Ampicillin) and grown at 37°C for 16 h. Single colonies were then picked and grown overnight with shaking. The plasmid DNA was isolated and double digested with the enzymes *Bam*HI and *Xho*I.

Analysis and Identification of the Recombinant Protein Expression

The pET32a-α-ε strain was grown in LB liquid medium (supplemented with 50 μg/mL Ampicillin) at 37°C and incubated in a shaker (Thermo MaxQ™ 6000) at 220 rpm until the optical density at 600 nm (OD_{600}) reached 0.6. After collecting 1 mL bacterial fluid as an un-induced reference, the protein expression was induced by addition of isopropyl-β-d-thiogalactopyranoside (IPTG) to the rest of the culture. The induced protein expression for the strain was conducted by using 1 m*M* of IPTG at 16°C and incubated for over 16 h. The bacterial fluids with and without being induced with IPTG were then collected for expression analysis. The specific steps are described in the followings. The bacterial fluids (1 mL) were collected and transferred into an appropriately marked centrifuge tube (1.5 mL). The bacterial fluid was centrifuged at 8,000 rpm and 4°C for 30 min. The bacterial precipitate was then obtained after the removal of the supernatant. The cells were then suspended in phosphated bufferd saline (PBS, 1 mL) and centrifuged at 8,000 rpm for 5 min, followed by the removal of the supernatant. The protein precipitate was washed and re-suspended in PBS (200 μL) and then sonicated until clearness. The attained fluid containing all bacterial proteins was centrifuged at 16,000 rpm and 4°C for 30 min. The supernatant and protein precipitate were collected. The protein precipitate was re-suspended in PBS (50 μL) then washed and precipitated. The all-bacterial-protein fluid, protein supernatant, and protein precipitate were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer (10 μL, 5 times) and completely mixed. Fractions of 6 μL were collected for SDS–PAGE analysis.

Optimization of the Temperature and Duration for the Induced Expression

Cultures of the pET32a-α-ε strain were grown in LB liquid medium supplemented with 50 μg/mL Ampicillin (obtained by adding Ampicillin in LB liquid medium until the concentration of Ampicillin reaches 50 μg/mL). The pET32a-α-ε strain cultures were incubated at 37°C in a shaker (Thermo MaxQ™ 6000) at 220 rpm until the OD_{600} value (taking the LB liquid medium with 50 μg/mL Ampicillin as reference) reached 0.6. The expression experiments were conducted with 1 m*M* of IPTG at 16°C over 8, 16 or 24 h.

Optimization of the IPTG Concentration

The expression experiments were conducted with 0.1, 0.3, 0.5, 0.75, 1 or 1.5 m*M* of IPTG at 16°C and incubated for 16 h.

Purification of α-ε Proteins

Nickel affinity chromatography followed by size-exclusion chromatography was implemented for purification of the His-tagged toxins. The entire-bacterial-protein supernatants were filtered by 0.45 μm filters and then applied to a HiTrap™ Chelating HP 10 mL column prepacked with a pre-charged Ni^{2+} column that was equilibrated with buffer 1 (20 m*M* Tris, 150 m*M* NaCl, water (solvent) and pH 8.0). Nickel affinity chromatography was performed using Fast Performance Liquid Chromatography (ÄKTA FPLC, GE Healthcare). After the protein was loaded onto the Ni^{2+}-chelating affinity column, it was washed with 10 bed

volumes of buffer 1 and 10 bed volumes of buffer 2 (20 m*M* Tris, 150 m*M* NaCl, 50 m*M* imidazole, water (solvent) and pH 8.0). The protein peak was monitored on ÄKTA FPLC. The recombinant protein on Ni-NTA was then eluted with buffer 3 (20 m*M* Tris, 150 m*M* NaCl, 300 m*M* imidazole, water (solvent) and pH 8.0). The eluted samples of the target protein peak were collected using ÄKTA FPLC and used as target protein samples after nickel affinity chromatography. The target protein samples were applied to Superdex 200-pg column for further purification. Buffer 1 was used as the running buffer for the column. Most imidazole was removed from the target protein samples after size-exclusion chromatography. The purified target protein samples were collected from the column by monitoring the protein elution profile. The protein (soluble target protein) concentration in the purified target protein samples was quantitatively acquired by NanoDrop™ 2000 UV-Vis Spectrophotometer (ND 2000). The protein concentration in the all-bacterial-protein fluid was also obtained by using ND 2000 and thus we determined the total bacterial protein concentration.

Preparation of the *C. perfringens* Vaccine

A α-ε protein solution (with concentration of 1000 μg/mL) was obtained for immunization test by dissolving the purified α-ε protein in sterile PBS. The α-ε protein solution was mixed with complete Freund's adjuvant (volume ratio 1:1) and followed by the process of emulsification. The resultant oil-emulsion vaccine will be used as the first vaccine candidate. The resultant oil-emulsion vaccine, obtained by mixing the α-ε protein solution with incomplete Freund's adjuvant (1:1) followed by emulsification, will be used as the second vaccine candidate. The control group (PBS) was also used for immunization with complete or incomplete Freund's adjuvant (volume ratio 1:1) followed by emulsification, will be used as the first or the second vaccine candidate.

Recovery and Culture of *C. perfringens* Strains

The *C. perfringens* virulent strains used in this study, *i.e*., type A (C57-10), type B (C58-5), type C (C59-4) and type D (C60-11), were provided by China Institute of Veterinary Drug Control. The anaerobic beef-liver broth (400 μL) was used to triturate the cells contained in the ampules, resulting in homogenized suspensions of the frozen bacteria. The bacterial suspensions were then added to the anaerobic beef-liver broth (containing beef extract peptone) at the volume ratio of 1:100. The ampules were sealed with 1 to 2-centimeter-thick liquid paraffin to prevent the contact with air. The prepared ampules of bacteria were placed in an anaerobic chamber and incubated at 37°C for 16–24 h. The growth of the bacteria was examined thereafter. After the process of smearing, staining, and microscopic examination and after one to two generations of propagation, the recovered *C. perfringens* was then ready for use. Fractions of the recovered *C. perfringens* were stored in 30% (v/v) glycerol-saline solution at -80°C.

***C. perfringens* Toxin Challenge**

The effect of α-ε proteins was examined against *C. perfringens* strains type A (C57-10), type B (C58-5), type C (C59-4) and type D (C60-11), respectively. Two hundred female Kunming mice (18–22 g in weight) were randomly assigned to two groups: one group with 160 mice for toxin challenge and the other group with 40 control mice for PBS reference. The first-time and second-time and third-time immunizations were conducted on the mice from the toxin-challenge group. The first-time immunization was done with the first vaccine candidate. The second and third-time immunizations were done with the second vaccine candidate. The immunized mice were administered with the dose level of 0.2 mL per mouse (α-ε protein 100 μg per mouse) each time. The control mice from PBS-reference group were injected with PBS in the presence of complete or incomplete Freund's adjuvant (volume ratio 1:1) with the level of 0.2 mL per mouse for the three times of immunization. Blood was collected from the mice before the first-time immunization. Serum was isolated from the collected blood for use as negative control. The second-time immunization was conducted 14 days after the first-time immunization, and the third-time immunization was performed 14 days after the second-time immunization. All mice from both groups were injected with varied types of *C. perfringens* two weeks after the third-time immunization, respectively. The specific level of injection is: 1.5×10^9 cfu for type A (C57-10), 2×10^9 cfu for type B (C58-5), 1.5×10^8 cfu for type C (C59-4) and 1.8×10^9 cfu for type D (C60-11).

Measurement of the Antibody Level in the Immunized Mice

Twenty 56-day-old female Kunming mice were immunized with α-ε protein, and titer of antibody against the target protein was detected at 7 days post-immunization and afterward it was continuously monitored for 84 days with an interval of about 7 days. Serum was isolated from the collected blood and was stored at -80°C for observation of the antibody level variation. Antibody titer was measured by indirect ELISA examination. The specific procedure of indirect ELISA is described in the followings. The ELISA plate was coated with the purified α-ε proteins. The concentration of the coating protein and the dilution of the serum were optimized based on checkerboard square matrix titration. The optimized concentration of the BSA blocking reagent was determined by applying BSA of varied concentrations. The working concentration and reaction time of the HRP-conjugated IgG were also optimized. The criterion: the optimized reaction condition corresponds to the highest P/N value (OD_{450} ratio of the positive to negative controls).

Based on the described indirect ELISA procedure, the antibody titer in the serum of the mice was measured for the period of 0-12 weeks after the first-time immunization.

Results

Analysis and Identification of the Protein Expression

The soluble α-ε target protein (75 kDa) was observed in the all-bacterial-protein fluid and supernatant of pET32a-α-ε induced with IPTG. Insoluble fraction was not found in protein sample isolated from strain pET32a-α-ε upon induction, as demonstrated in Fig. 1.

Purification of α-ε Proteins

It was shown that the α-ε target proteins based on pET32a-α-ε are mostly soluble and exist in the broken-cell supernatant fluid. The control band of the soluble expression of the protein is clear. The amount of impurities in the supernatant is relatively low. With optimization of the nickel affinity chromatography procedure (ÄKTA FPLC), a higher level of purity can be achieved in the soluble target protein band. Most imidazole can be removed from the target protein samples after further purification with size-exclusion chromatography. The purified soluble protein can be employed in the preparation of diagnostic antigen, monoclonal antibody, or genetically engineered subunit vaccine.

The amount of the expressed α-ε protein will increase as the temperature is lowered. The maximal amount of α-ε protein can be reached under the condition of 16°C for 16 h. There will be a slight decrease in the expressed α-ε protein if the duration is extended to 24 h. Therefore, it was experimentally confirmed that the optimal condition for the induced expression on pET32a-α-ε is 16°C for 16 h.

Optimization of IPTG Concentration

The results showed that the amount of the expressed protein varies with the IPTG concentration. The amount of the expressed α-ε protein is proportional to the IPTG concentration when it varies between 0.1 – 1.5 m*M*. There is a decrease in the amount of the expressed protein when the IPTG concentration is 1.5 m*M*. This may be attributed to the toxicity of IPTG. As such, the optimal IPTG concentration was determined as 1 m*M* for the induced expression.

Result of Toxin Challenge Test

The toxin-challenge result indicated that certain levels of protection were developed in the α-ε immunized mice against *C. perfringens* type A through D, respectively. The immunized mice got protected against *C. perfringens* type A, B, C and D with the survival rate of 100%, 90%, 85% and 100%, respectively. All mice in the PBS-reference groups died. A chart is provided in Table 1 demonstrating the survival rate in the immunized group under four types toxin challenge.

Table 1: The survival rate after challenge

Strain type for challenge	Number of mice in immunization group	Number of mice in control group	Survival rate (survived/immunized)	
			Immunization group	Control group
A	40	10	100% (40/40)	0% (0/10)
B	40	10	90% (36/40)	0% (0/10)
C	40	10	85% (34/40)	0% (0/10)
D	40	10	100% (40/40)	0% (0/10)

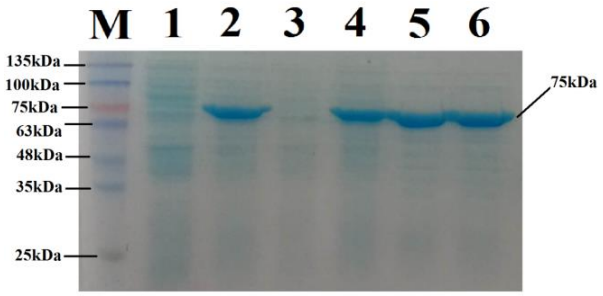

Fig. 1: SDS-PAGE analysis of the purified protein
M: Protein Marker (molecular weight, up to down: 135 kDa, 100 kDa, 75 kDa, 63 kDa, 48 kDa, 35 kDa, 25 kDa); Lane 1: Protein sample isolated from strain pET32a upon induction (negative control); 2:Protein sample isolated from strain pET32a-α-ε upon induction (soluble and insoluble fraction); 3:Protein sample isolated from strain pET32a-α-ε conducted with 1 m*M* of IPTG at 16 °C and incubated for 16 hours (insoluble fraction); 4: Protein sample isolated from strain pET32a-α-ε conducted with 1 m*M* of IPTG at 16°C and incubated for 16 hours (soluble fraction); 5: α-ε protein (soluble fraction) purified by nickel column; 6: α-ε protein (soluble fraction) purified by column molecular sieve

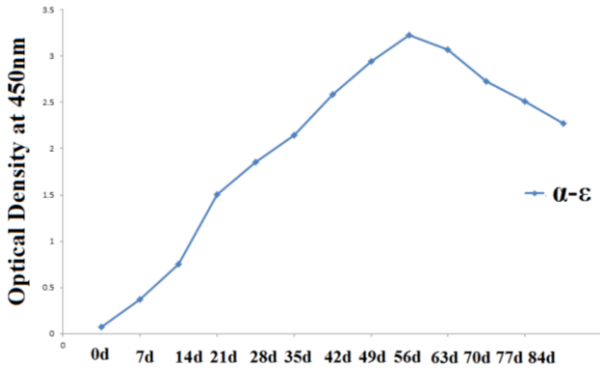

Fig. 2: Kinetic titration of antibody of the immunized mice
Twenty 56-day-old female BALB/C mice were immunized with α-ε protein, and titer of antibody against the target protein was detected at 7 days post-immunization, and afterward it was continuously monitored for 84 days with an interval of about 7 days

The Level of the Antibody Raised Against the Candidate Vaccine

The purified α-ε protein was used as the diagnostic antigen coated to the ELISA plate. Measurement was done for the immune antigen of the mice or the antibody titer in the

serum after the toxin challenge. It was found that remarkable sensitivity and specificity can be achieved in the measurement by employing the α-ε protein as the diagnostic antigen. The maximum P/N value was achieved with 1.5 μg/mL of α-ε protein and serum dilution being 1:50. Therefore it is determined that the optimal coating-antigen concentration is 1.5 μg/mL and the optimal serum dilution is 1:50. By setting the concentration of HRP-conjugated goat anti-mouse IgG as 1:50000 at 37°C for 1 h, an optimal OD_{450} value can be achieved 12 min after the addition of TMB solution. The antibody titer in the serum of 20mice, which were in a separate group, intended only for monitoring the kinetic of immune response, was measured for a 12 week period after immunization. A remarkable rise in the level of antibody titer was observed in the mice immunized with the α-ε fusion toxin protein. Specifically, the level of antibody titer rapidly increased 7 days after immunization and reached the peak 8–9 weeks after immunization. The highest level was kept until a slight decrease was noticed 10 weeks after immunization, as demonstrated in Fig. 2.

Discussion

In early 1980s, monovalent vaccines and inactivated multivalent vaccines against *C. perfringens*-associated diseases were successfully developed in China. These developed vaccines played a significant role in providing protection against diseases associates with *C. perfringens*. But, during the process of vaccine manufacturing, the yield of toxins highly depends on the culture time, temperature, inoculation amount and the quality of the culture medium. Moreover, problems, such as low safety level and poor stability, are present in traditional vaccines (Ewoldt and Anderson, 2005; Songer and Miskimins, 2005; Lebrun, 2007; Lebrun *et al.*, 2010). As such, it's become a key objective for world-wide researchers to develop multi-serotype genetically engineered subunit multivalent vaccines.

In this study, an efficient expression of soluble antigen in *E. coli* was successfully achieved through a series of research efforts, *e.g*., conducting codon optimization, removing the signal peptide, selecting sequences of higher hydrophilicity and antigenicity, removing the lethal gene and optimizing the expression conditions. etc. The *E. coli* bacteria are of high expression level at low cost of production. The success in efficient expression of active proteins in *E. coli* provides a solid foundation for further development of genetically engineered subunit vaccines. Based on the efficient expression of recombinant α-ε-toxin protein, we developed a fusion protein vaccine for inhibition of the infections by *C. perfringens.* A relatively high level of antibody was detected in the serum of the animals immunized by the developed vaccine. Protection was provided by the vaccine against the toxin challenge of *C. perfringens*. The vaccine is able to protect the immunized mice against *C. perfringens* type A, B, C and D with the survival rate of 100%, 90%, 85% and 100%, respectively.

Genetically engineered subunit vaccines processed advantages such as high level of safety, improved stability, high purity and high yield. In the meanwhile, multiple types of toxins can be combined through genetic engineering to construct new toxin proteins for expression (Chandran *et al.*, 2010; Langroudi *et al.*, 2011; Wang *et al.*, 2011). Although great efforts were spent by researchers on expression and purification of major *C. perfringens* exotoxin proteins, the expressed toxin proteins are generally all inactive inclusion bodies. Highly efficient expression and purification of soluble and bioactive exotoxin proteins have not been reported. Tried to fuse the genes of *C. perfringens* epsilon toxin, and partly obtained the expression of fusion proteins with similar biological activity of the native protein. The detoxified beta fusion toxoid protected up to 90% of immunized mice against experimental challenge (Lobato *et al.*, 2010; Mathur *et al.*, 2010; Pilehchian *et al.*, 2013). The vaccines were prepared by adding adjuvant to a number of recombinant fusion toxins that were inactivated with formaldehyde (Zeng *et al.*, 2011). These developed vaccines together with goat (sheep) pox vaccines were employed to immunize goats (sheep). The results confirmed that the immunized goats (sheep) obtained immune protection against both goat (sheep) pox and enterotoxaemia. Cloned toxin gene into associated plasmid and then transformed the plasmid into *E. coli* competent cells (Pilehchian *et al.*, 2013). The recombinant toxin protein was inactivated and mixed with associated adjuvant, and then employed to immunize livestock ruminants (Miyamoto *et al.*, 2008). Increase of the antibody titer was observed in the animals after the second-time immunization. It was measured that the recombinant toxin is able to provide a certain level of immune protection against *C. perfringens.* Gong *et al.* (2015) constructed the recombinant plasmid encoding the fusion genes of α-, β2- and ε-toxins. However, the α-ε-β2 protein expressed with the recombinant plasmids were inclusion bodies. In addition, they did not test the immonogenecity of the recombinant protein in animal studies (Gong *et al.*, 2015).

The *E. coli* bacteria are of high expression level at low cost of production. The success in efficient expression of active proteins in *E. coli* provides a solid foundation for further development of genetically engineered subunit vaccines. For the first time we constructed the expression vector pET32a-α-ε for the multi-toxin fusion protein. The efficient expression and purification of the soluble target protein was successfully achieved. The expressed multi-toxin fusion protein exhibited remarkable immunogenicity.

Conclusion

The expressed protein can be utilized in the development of genetically engineered trivalent subunit vaccines against animal diseases caused by *C. perfringens* infections, *e.g.*,

braxy, ovine enterotoxaemia, lamb dysentery gas and sheep struck, and it shows promising application potentials and prospects.

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