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Expression of *Clostridium perfringens* epsilon-beta fusion toxin gene in *E. coli* and its immunologic studies in mouse

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ABSTRACT

Clostridium perfringens is an anaerobic spore-forming, pathogenic bacterium that is responsible for severe diseases in humans and livestock. In the present study, an epsilon-beta fusion toxin was expressed as a soluble protein in *E. coli* and the recombinant cell lysate was used for immunization studies in mouse. Potency of the toxin (as an antigen) induced 6 and 10 IU/ml of epsilon and beta anti-toxin in rabbit, respectively. These titers were higher than the minimum level required by the European Pharmacopoeia for epsilon and beta toxins. Experimental challenge with the recombinant fusion toxoid revealed that it could protect mice against *C. perfringens* epsilon and beta toxins. Toxicity of the fusion toxin was studied by histopathological findings, which were the same as the native toxins. In conclusion, *E. coli* is a suitable expression host for immunogenic epsilon-beta fusion toxin of *C. perfringens.*

1. Introduction

Clostridium perfringens (*C. perfringens)* is a Gram-positive, rod-shaped, anaerobic, spore-forming, and heat-resistant bacterium of genus *Clostridium* [1]. It produces numerous toxins which are responsible for severe diseases in humans and livestock. It is also a secondary pathogen in diseases, such as necrotic enteritis [2,3]. *C. perfringens* is classified into five isotypes (A, B, C, D, and E) based on producing four major toxins, iota (*iA*), alpha (*cpa*), beta (*cpb*) and epsilon (*etx*) [4,5].

Epsilon toxin is a major lethal toxin which is produced by *C. perfringens* types B and D. It is responsible for a rapidly fatal enterotoxemia in economically important livestock [4].

Beta toxin is produced by *C. perfringens* type B and type C and causes fatal diseases originating in the intestines of humans or livestock [6–8]. It is known to aid in the lysis of endothelial cells by forming pores in the cell membrane [9]. This function is necessary for both necrotizing enteritis and lethal enterotoxemia caused by type C isolates [10,11].

Vaccination seems to be the most effective way to control *C. perfringens* diseases. However, the industrial production of Clostridial toxins is laborious [12]. Therefore, the production of toxins in heterologous expression systems is a viable alternative. The efficiency of vaccines based on Clostridium recombinant toxins has been reported [13–16]. It has been shown that recombinant beta and epsilon toxins expressed in *E. coli* induce appropriate immune responses in mice and ruminants [16–18].

Recombinant DNA technology allows for fusion of genes or gene fragments in a simple manner. Fusion of Clostridial toxins has been reported in *E. coli* expression system [19–21]. In our previous study, a genetic construct containing *C. perfringens* toxin genes, epsilon and beta, was fused using a small linker sequence [22,23]. In this study, the epsilon-beta fusion toxin was expressed in *E. coli* and its immunogenicity was studied in mouse.

2. Materials and methods

2.1. Construction of the fusion gene of epsilon- and beta-toxins

Culture of *C. perfringens*, extraction of genomic DNA and construction of pJETεβ plasmid containing the epsilon-beta fusion gene were described in a previous study [22].

2.2. Expression of the recombinant protein

The pJETεβ and pET22b plasmids were digested by *Nde*I and *Xho*I and then gel-purified fragments including fusion gene and pET22b were ligated and transformed into *E. coli* strain TOP10. After confirming the recombinant pET22εβ plasmid by restriction enzyme

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digestion and sequencing, the plasmid was transformed into *E. coli* strain Rosetta.

The protein expression was performed by the culture of bacteria in LB medium containing selective antibiotic at 37 °C to OD_{600} 0.65–0.75. The protein expression was induced by adding 0.5 mM IPTG and then the growth was continued for 22 h. The samples were collected at different times after induction. After centrifugation, the bacterial pellet was stored at −80 °C for SDS-PAGE and Western blot analysis.

To optimize the expression conditions, the bacterial culture was induced by different concentrations of IPTG. Also, the effect of temperature on protein expression level was studied at 25, 31 and 37 °C.

2.3. Purification of the fusion protein

The recombinant fusion protein, which contains a C-terminal His tag, was purified by Ni-NTA resin. The bacterial pellet was suspended in lysis buffer (NaH_2PO_4 50 mM, NaCl 300 mM, and imidazole 10 mM, pH 8) and the cells were lysed by 6 times sonication on ice for 5 min (each pulse duration was 40 s and 30 s intervals). The cell lysate was centrifuged at 13,680 × *g* and the clarified supernatant was loaded on Ni-NTA resin at the flow rate of 1 ml/min. The column was washed with 5 volumes of wash buffer (NaH_2PO_4 50 mM, NaCl 300 mM, and imidazole 20 mM, pH 8) and finally the protein was eluted by adding elution buffer (NaH_2PO_4 50 mM, NaCl 300 mM, and imidazole 250 mM, pH 8). The purified protein was analyzed using SDS-PAGE and Western blot. The protein concentration was determined using a standard procedure as described previously [24].

2.4. Detoxification and residual toxicity test

Detoxification was done by incubation of recombinant cell lysate (*E. coli* cells expressing the beta-epsilon fusion protein) and *C. perfringens* types B and D culture, with 0.6% formaldehyde (Merck) and incubation at 30 °C for at least a week. Residual toxicity was determined according to the European Pharmacopoeia [25]. Briefly, 0.5 ml of detoxified toxin was intravenously inoculated into ten NMRI mice with body weight of 18–20 g. Lack of mortality in the mice 72 h post-inoculation indicates the absence of toxicity (toxoid).

2.5. Serum neutralization test

A group of ten Dutch rabbits, three- to six-month-old, were injected with 1 ml of detoxified recombinant cell lysate (SC). Four weeks later second injection of the same dose was given. Two weeks later, the blood samples were collected and the sera were pooled together (RPS). The units of epsilon and beta antitoxins in RPS were estimated by titration in 40 NMRI mice with body weight of 18–20 g. For this purpose, 20 mixtures in four groups were prepared using epsilon or beta standard antitoxins and the related diluted RPS. Two mice were injected with 0.5 ml of one of the mixtures and observed for 72 h. Based on the mouse mortality, epsilon or beta antitoxin amounts were calculated [25].

2.6. Mouse immunization

Fifty NMRI mice with the body weight of 18–20 g were randomly divided into five equal groups. The first experimental group was injected (IV into the lateral tail vein) with 0.5 ml of non-detoxified recombinant cell lysate (toxin). The second group received 0.5 ml of detoxified recombinant cell lysate (toxoid). In the third group, each mouse was injected with 0.5 ml of non-detoxified *C. perfringens* types B and D culture. In the fourth group, each mouse was injected with 0.5 ml of detoxified *C. perfringens* types B and D culture, (positive controls). Mice in the fifth group were injected with 0.5 ml active non-recombinant cell lysate as negative control. Four weeks later, the survived mice were challenged by injection of 0.5 ml non-detoxified culture of *C. perfringens* types B and D. The ratio of the survived mice to the total number of mice was calculated during a week. The different organs such as intestines, kidney, liver, lung, heart, spleen, and brain of mice in experimental and negative control groups were examined using the light microscopy.

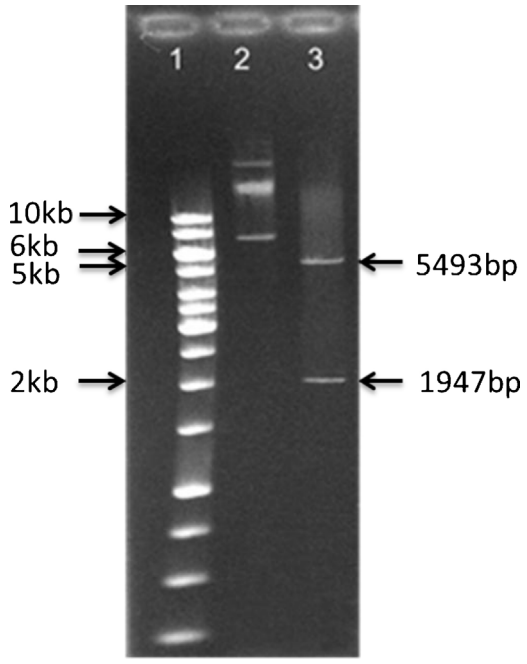

Fig. 1. Construction of pET22bεβ expression vector. Lane 1: DNA molecular weight markers; lane 2: uncut pET22bεβ plasmid; lane 3: digested pET22bεβ vector.

3. Results

3.1. Expression of the fusion protein of epsilon and beta toxins

Previously, epsilon and beta toxin genes were fused and cloned in pJET1.2/blunt vector to obtain pJETεß plasmid (Pilehchian Langroudi et al. [22]). Sequencing analysis revealed that the fusion gene length is 1947 bp, where nucleotides 1 to 984 belong to epsilon toxin gene with its signal peptide sequence, nucleotides 985 to 1020 (36 bp) are a linker sequence which is optimized for *E. coli*, and nucleotides 1021 to 1947 (927 bp) belong to beta toxin gene without its signal peptide sequence. *Nde*I restriction site and its flanking region at the 3′ end of epsilon gene sequence, and *Xho*I restriction site and its flanking region for the 5′ end of beta gene sequence are also present. This sequence was deposited in GenBank (accession number JF833085). In this study, the pJETεβ plasmid was digested with appropriate restriction enzymes and a 2 kb fragment corresponding to epsilon-beta fusion toxin gene was inserted into pET22b plasmid linearized with the same enzymes (Fig. 1).

SDS-PAGE analysis revealed that the epsilon-beta fusion protein is expressed 2 h after induction by 0.5 mM IPTG, and continued up to 22 h later (Fig. 2). No difference was observed in the protein expression level using IPTG gradient. But the effect of temperature on the protein expression level was significant, in that the best expression was detected at 37 °C (Fig. 3). The recombinant fusion protein containing a histidine tag at the C-terminus was purified by Ni-NTA resin and the purified protein appeared as a ∼72 kDa protein band on SDS-PAGE and Western blot (Fig. 4).