

Rare codons optimizer host strain of *E. coli* improves expression of *Clostridium septicum* alpha toxin gene

Abstract

Aims: *Clostridium septicum* is an anaerobic, gram positive bacterium that is able to form resistant spores. It produces numerous toxins that cause many diseases such as gas gangrene in humans and braxy in farm animals. In the present study, an attempt was done to express an active fragment of alpha toxin gene of *C. septicum* vaccine strain, in *E. coli* strains BL21 (DE3) and Rosetta (DE3) strains.

Methodology: In the present study, the active fragment of alpha toxin gene of *C. septicum* vaccine strain was amplified and ligated with pJET1.2blunt cloning vector and after cloning was extracted from the pJETasep recombinant cloning vector. After purification and evaluation, it was ligated with pET22b(+) expression vector and the pET22asep was transformed into *E. coli* strains BL21(DE3) and Rosetta(DE3) strains.

Results: Results showed that a recombinant protein was expressed as a soluble protein after IPTG induction in Rosetta (DE3) rare codons optimizer host strain, but not in BL21 (DE3). Further optimization on expression conditions of the recombinant alpha toxin protein was achieved by incubating the culture of recombinant *E. coli*/Rosetta (DE3[pET22asep]) cells in 37°C and induction with 0.5, 1.0 and 1.5 mM IPTG for 3-6 h. Protein expression was evaluated by SDS-PAGE and the recombinant alpha toxin protein was purified using Ni-NTA resin and for its reconfirmation was analysed by Western blot.

Conclusions: We concluded that, *E. coli* strain Rosetta (DE3) is a suitable expression host for production of *C. septicum* alpha toxin and the obtained recombinant plasmid could be used for further research on production of recombinant vaccine against braxy disease.

Keywords: alpha toxin, expression, rare codons, Rosetta

1. Introduction

Clostridium septicum is a large anaerobic, Gram positive, rod shaped and fermentative bacterium. Peritrichous flagella enable the bacterium to be motile and its drumstick-like shape is because of its terminal spore. It is a member of the normal gut flora in humans as well as farm animals [1]. *C. septicum* can produce and secrete numbers of toxic proteins such as beta, gamma, delta and alpha which is hemolytic, lethal and necrotizing toxin. There are several reports about *C. septicum* which is responsible for some diseases, the most serious are "frequently fatal non-traumatic gas gangrene" and "cancer" [2, 3] in human and braxy, malignant oedema and black quarter in farm animals. Immunity to these diseases is mediated mainly by antitoxin raised against *C. septicum* alpha toxin [4].

The activity of *C. septicum* alpha-toxin had been determined in erythrocytes of various animals and it had been cleared that the sensitivity is in the order of mouse, rat, canine, equine, rabbit, chicken, bovine, swine and ovine. This report concluded that, alpha-toxin associates with specific erythrocyte membrane proteins in any animal species, and is subsets of glycosylphosphatidylinositol-anchored proteins in various animal species [5]. Alpha-toxin is functionally similar to aerolysin and is a beta-barrel pore-forming cytotoxin. Those residues which are important in receptor binding, oligomerization, and pore formation, have been identified. Although it is obvious that alpha toxin is essential for disease, but little is known about its activity in an infection [6].

Alpha toxin is a cytolytic protein with a molecular mass of approximately 48 kDa [7]. Alpha-toxin gene of *C. septicum* BX96 was cloned and expressed in *E. coli*. The toxin had 443 amino acids in length which its 31-residue signal peptide was removed from the toxin during secretion. There were no extended hydrophobic regions in the mature toxin sequence. It was determined that the alpha-toxin proteolytic activation located on the carboxyl-terminal side of arginine 398 [8]. Another report revealed that eukaryotic protease; furin is involved in the activation of *C. septicum* alpha-toxin on the cell surface but that alternate eukaryotic proteases such as trypsin can also activate the toxin. Regardless of the

activating protease, the furin consensus site appears to be essential for the activation of alpha-toxin on the cell surface [9].

In 1994 one fragment of DNA (2293 bp) as *C. septicum* alpha toxin gene was reported and deposited in GenBank with accession number D17668.1 [10]. Further studies on this fragment showed that the fragment consist of nucleotide numbers 561 up to 1982 (1332 bp) was active alpha toxin gene [8]. In the present study, attempt was done to express active fragment of alpha toxin gene of *C. septicum* vaccine strain, in *E. coli* strains BL21 (DE3) and Rosetta (DE3). Purified protein was analysed to show the efficacy of these hosts for this purpose.

2. Materials and Methods

Linearized pJET1.2/blunt cloning vector, *Pfu* DNA polymerase, dNTPs, T4 DNA ligase, *Nde*I and *Xho*I restriction enzymes and protein weight markers (PageRuler™), 100 bp Plus DNA size markers (GeneRuler™), and plasmid extraction kit were purchased from Fermentas. High Pure PCR Product Purification Kit for DNA fragments recovery was purchased from Vivantis. Sheep primary antibody and conjugate anti-sheep HRP were purchased from DAKO Company (Denmark)

2.1. Cloning

C. septicum vaccine strain (CN913), *E. coli* strain TOP10 (cloning host) and strain (BL21 (DE3) and Rosetta (DE3)) as expression host prepared from Razi Institute. *C. septicum* were cultured anaerobically using anaerobic chambers in liver extraction media at 37°C during overnight. After centrifugation supernatant was discarded and whole genomic DNA was extracted from pellet by phenol-chloroform method. Alpha toxin gene was amplified using one pair of specific primers consisting *Nde*I at the 5' end of forward and *Xho*I at the 3' end of reverse primers. These primers produce 1332 bp fragments of DNA that is only consisting of activated fragment of *C. septicum* alpha toxin. *Pfu* DNA polymerase was used for amplification of blunt-end PCR product. After ligation, pJETasep recombinant vector was transformed into *E. coli*/Top10 competent cell and screening of recombinant clones was done by antibiotic resistance and Colony PCR and nucleotide sequencing was carried out. The whole procedure was done as described previously [11].

2.2. Sub cloning

pJETasep was extracted from *E. coli*/TOP10/pJETasep clone and purified, then was digested by *Nde*I and *Xho*I to produce sticky-ends alpha toxin gene. After digestion and electrophoresis, the *csa* was extracted from the gel and purified. pET22b(+) as expression vector was digested using the same enzymes, then purified and ligated with *csa* active fragment.

E. coli strains BL21 and Rosetta were selected as expression host. After preparing of competent cells, pET22asep was transformed into competent cells, so *E. coli*/BL21/pET22asep and *E. coli*/Rosetta/pET22asep were provided. At the next step pET22asep recombinant expression vector was purified and digested using the same restriction enzymes.

2.3. Expression of alpha toxin

Confirmed recombinant cells were cultured in LB-Amp media and incubated at 37°C to OD₆₀₀=0.6-0.7. 0.5 mM IPTG was added to induce the protein expression and growth was continued for 18 h. Different concentration of IPTG (0.5, 1.0 and 1.5mM) was used for optimization of protein expression. Protein analysis was performed by SDS-PAGE.

2.4. Purification of the CSA

The recombinant CSA, which contains a 6-His tag at C-terminus, was purified by Ni-NTA resin. For this purpose the culture of recombinant cell was centrifuged and the pellet was suspended in lysis buffer and the cells were lysed by 6 times sonication on ice for 5 min. 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 and finally the protein was eluted by adding elution buffer as described previously [12]. The purified protein was analysed using SDS-PAGE and Western blot. The protein concentration was determined using a standard procedure [13].

3. Results

Electrophoresis result showed that genomic DNA is extracted and PCR analysis revealed that blunt end *csa* is amplified. Recombinant pJETasep was produced and successfully transformed into *E. coli*/TOP10 competent cell. pJETasep was extracted from *E. coli*/TOP10/pJETasep cells and was digested by *Nde*I, *Xho*I enzymes so sticky-end *csa* was produced.

pET22b(+) was digested by the same restriction enzymes to produce sticky ends and was ligated with

sticky ended *csa*. pET22αsep recombinant expression vector was successfully transformed into *E. coli* strains BL21 and *Rosetta*. Recombinant *E. coli* strains were grown on LB-Amp agar and positive colonies were screened. Colony PCR was done to show the presence of recombinant pET22αsep vector into both recombinant *E. coli* colonies. Those colonies which showed *csa* (Fig. 1) were selected for the next step. pET22αsep was extracted from both recombinant expression hosts, then was digested and recombinant *csa* was shown (Fig. 2). Sequencing also revealed that insert gene is consistent with *csa* in GenBank. After expression, SDS-PAGE analysis showed that recombinant protein is well expressed 4 h after induction with 0.5 mM IPTG in recombinant *E. coli/Rosetta* (Fig. 3), cells but not in *E. coli/BL21*(Fig. 4). Different concentrations of IPTG had no significant effect on protein expression (Fig. 5). Recombinant protein was purified by Ni-NTA resin and result was shown on SDS-PAGE. Purified protein appeared as about 41.5 kDa protein band on SDS-PAGE and Western blot using sheep primary antibody and conjugate anti-sheep HRP (Fig. 6).

4. Discussion

C. septicum produces lethal antigen only in low titer and in a variable way. In addition, the immunogenicity of native toxic filtrates is weak, which results in poor antibody response in animals. CSA that is the lethal cytolytic and the major virulence factor, appears to be its immunodominant extracellular antigen [14]. Most commercial vaccines are inactivated and may contain combinations of clostridial bacterin, toxoid or bacterin/toxoid. Enough time is essential for provision of maximal protection at the most likely age of susceptibility. Genetic engineering helps us to produce recombinant protein that would be a good alternative to native toxins of *C. septicum* [15]. This bacterium produces several extracellular factors, but CSA is the only toxin that is secreted as a single lethal extracellular toxin [16].

In 1995 expression of alpha-toxin in *E. coli* BL21 resulted in the production of a 46.5 kDa inactive protoxin (ATpro) of, which was identical to *C. septicum* native toxin and requires proteolytic activation to yields a cytolytically active form (ATact) 41.3-kDa. This report showed that it respect to activity and activation[8]. The activation also could be done *in vitro* using trypsin, that nicks the toxin at R367, near the C-terminus [7]. Another report demonstrated that the activation leads to oligomerization into a prepore complex [17], which subsequently inserts into the membrane to form the beta barrel pore. The pore has been estimated to be approximately 1.6 nm in diameter [7].

Based on this information, we decided to clone and express a restricted fragment of the *csa* that could lead to the 41.5 kDa recombinant active CSA toxin.

In the case of *E. coli*, a clear codon bias exists among the 61 amino acid codons found within the population of mRNA molecules, so this situation can cause serious problems after the initiation of transcription of a cloned heterologous gene in the *E. coli* host. Some reports suggest clusters of AGG/AGA, CUA, AUA, CGA or CCC codons can reduce both the quantity and quality of the synthesized protein. An excess of any of these codons, may create translational problems. *E. coli* strain *Rosetta* is a good expression host for alpha toxin because it is designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli* and supplies tRNAs for above mentioned codons, on a compatible chloramphenicol-resistant plasmid [18].

In the present study, recombinant plasmid pET22b(+) carrying *C. septicum csa* was transformed into *E. coli* strain *Rosetta*. Sequencing result revealed that insert gene was 1332 bp. However our study showed that BL21 cannot express *C. septicum* CN913.alpha toxin with its rare codons.pET22b (+) showed to be a suitable vector for expression of alpha toxin.

In our work, recombinant toxin expression was started 30 min. after induction with IPTG and continued for 18 h. After considering of different concentrations of IPTG and different temperatures it was revealed that different concentration of IPTG has no obvious effect on protein expression level. Although we could not find any report regard to IPTG concentration effects on *csa* expression, but a previous study showed that IPTG gradient has no effect on expression of epsilon-beta fusion gene [12]. We also fund that optimal thermal condition for alpha toxin protein expression is 37°C. At the present, accessing recombinant bacterium and manufacturing of recombinant vaccine is possible. Therefore prevention of clostridial diseases would be happen by manufacturing of vaccine in safe conditions and less cost.

5. Conclusions

Finally we concluded that, *E. coli/Rosetta* is a suitable expression host for production of *C. septicum* alpha toxin and this clone could be used for further studies on production of braxy recombinant vaccine.

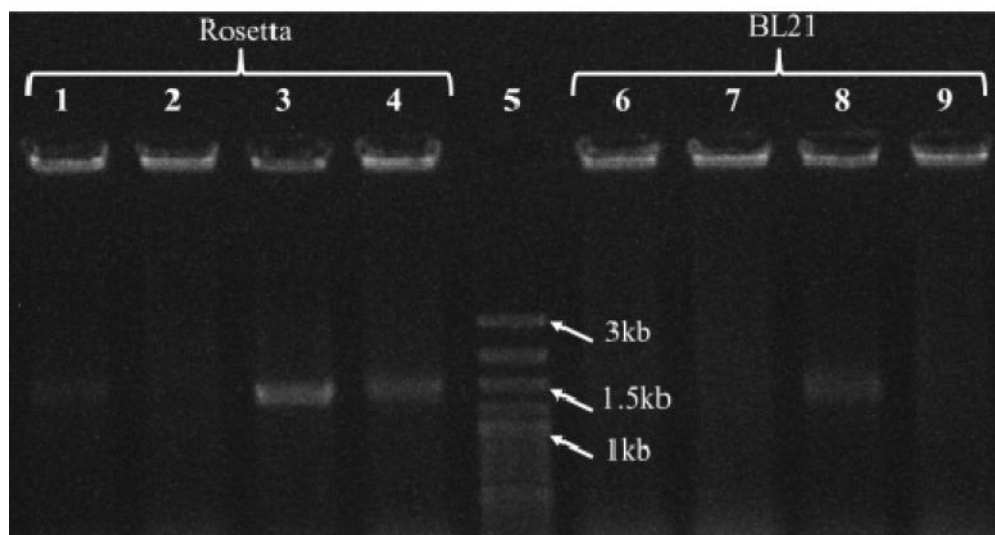


Fig.1. Colony PCR analysis of two different recombinant strains of *E. coli*. Lanes 1-4; *E. coli*/Rosetta/pET22asep, lanes 6-9; *E. coli*/BL21/pET22asep. Alpha toxin gene (about 1332 bp) could be seen in lanes 1, 3, 4 and 8. Lane 5; 100 bp plus DNA molecular size markers.

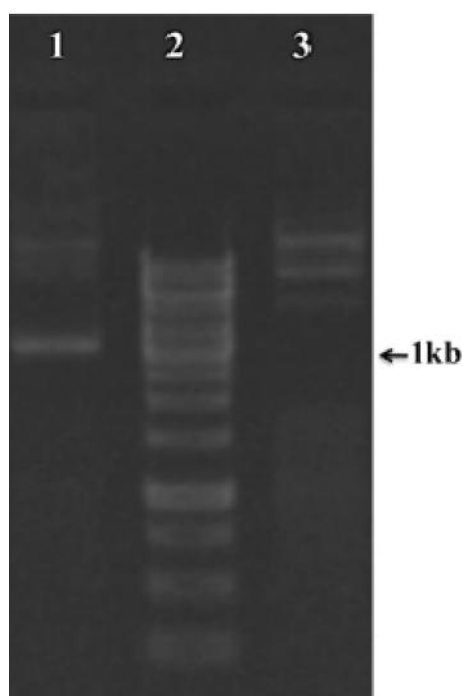


Fig. 2. Agarose gel electrophoresis of pET22asep recombinant expression vector digestion. Lane 1; digested pET22asep showing alpha toxin gene (*csa*), lane 2; 100 bp plus DNA molecular size markers, lane 3; undigested pET22asep.

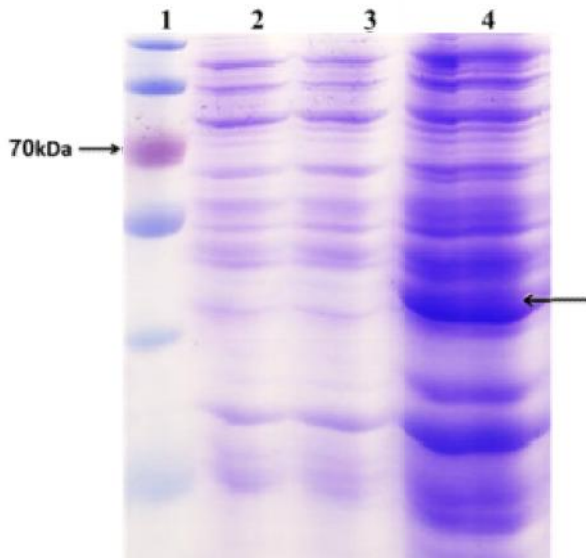


Fig. 3. SDS-PAGE analysis of alpha toxin expression. Lane 1: protein weight markers (PageRuler), Lane 2; *E. coli*/Rosetta/pET22b(+) (Negative control). Lane 3; *E. coli*/Rosetta/pET22asep without induction, Lane 4; *E. coli*/Rosetta/pET22asep 4 h after induction with 0.5 mM IPTG, arrow shows recombinant alpha toxin protein (CSA).

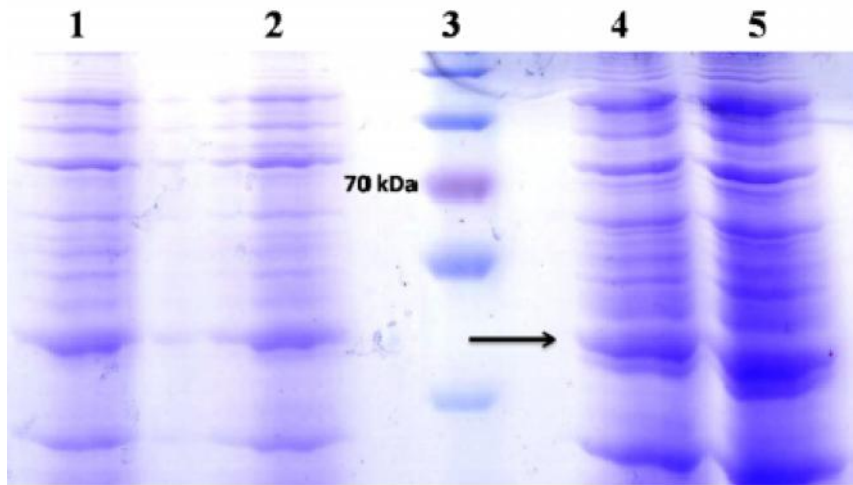


Fig 4. Comparison of alpha toxin expression in *E. coli*/BL21/pET22β and *E. coli*/Rosetta/pET22β. Lanes 1 and 2; *E. coli*/BL21/pET22b(+), lanes 4 and 5; *E. coli*/Rosetta/pET22β, 4 h after induction with 0.5 mM IPTG, arrow shows recombinant alpha toxin protein (CSA). Lane3 protein weight markers (PageRuler).

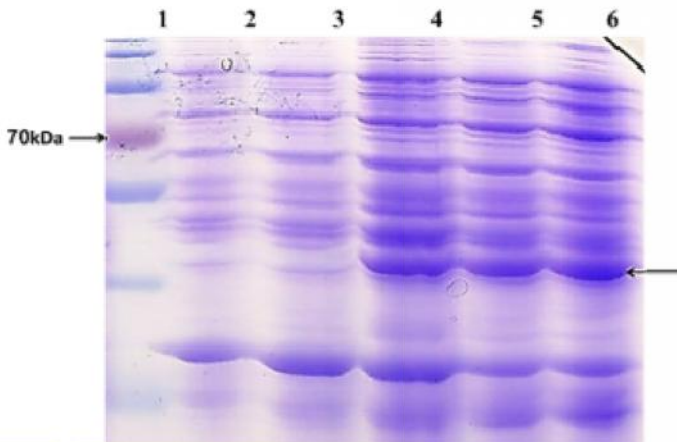


Fig. 5. Effect of IPTG concentrations on expression: lane 1; protein weight markers (PageRuler), lane 1; (Negative control), lane 3; *E. coli* Rosetta/pET22αsep without induction, Lanes 4-6; *E. coli* Rosetta/pET22αsep 4 h after induction with 0.5, 1.0 and 1.5 mM IPTG, arrow shows recombinant alpha toxin protein (CSA).

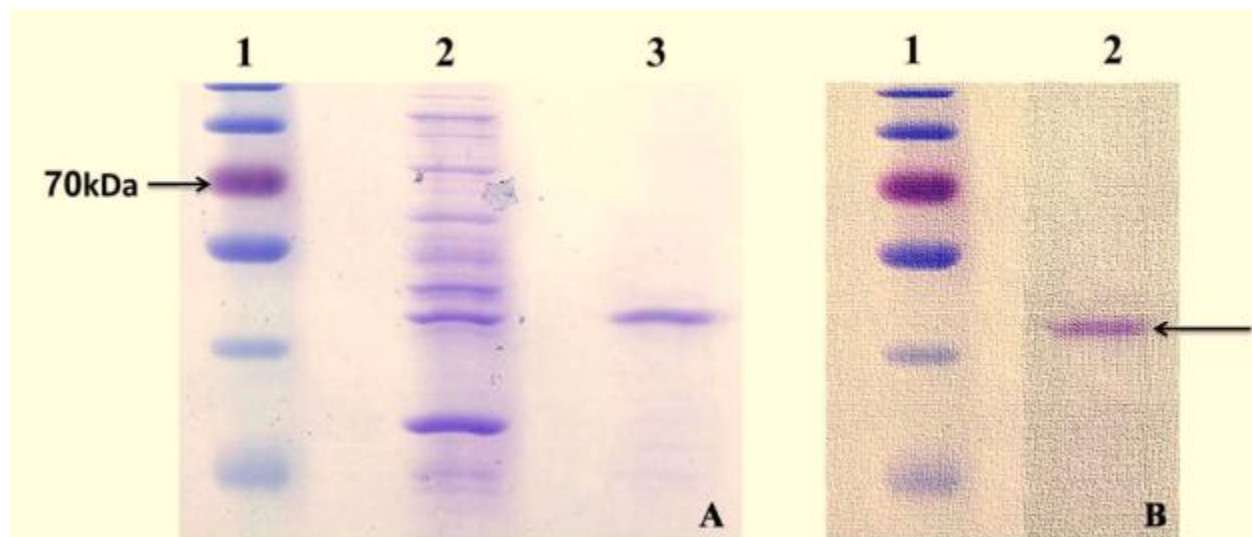


Fig. 6. A) SDS-PAGE, lane1; protein weight markers (PageRuler), lane 2; *E. coli* Rosetta/pET22αsep 4 h after induction with 0.5 mM IPTG at 37°C. Lane 3; purified recombinant alpha toxin (CSA). B) Western blot analysis, lane 1; protein weight markers (PageRuler), Lane 2; purified recombinant alpha toxin.

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