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Recombinant Expression and Purification of *Pseudomonas aeruginosa* Truncated Exotoxin A in *Escherichia coli*

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ABSTRACT

Background: *Pseudomonas* (*P.*) *aeruginosa* exotoxin A (PE) is one of the most potent bacterial toxins ever been identified. It catalyzes ADP-ribosylation of cellular elongation factor 2 and specifically arrests protein synthesis leads to cell death. Different derivatives of PE have been used for construction of immunotoxins against cancers. The aim of this study was to clone and express a DNA fragment of PE encoding a truncated exotoxin lacking the cell binding domain of native exotoxin. ***Methods*****:** The genomic DNA extracted from *P. aeruginosa* PAO1 was used in a polymerase chain reaction (PCR) containing the primers for amplification of domains 2-3 of PE gene. The PCR product was then cloned into the pET-22b vector and transformed into *E. coli* BL21 cells for expression. The His-tagged recombinant PE38 was purified by Nickel affinity chromatography and characterized. ***Results*****:** Sequencing of the cloned fragment confirmed the identity of gene as PE domains 2-3. Analyzing the recombinant protein expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed high level expression of recombinant protein. Results of western blotting with anti native-exotoxin A showed proper conformational structure of purified recombinant protein. ***Conclusion***: The results of this study indicated that the presented expression system is an efficient system for the production of recombinant truncated exotoxin A. This recombinant protein can be used for the construction of toxin conjugates against different cancers.

Introduction

Pseudomonas aeruginosa exotoxin A (PE) is one of the most potent toxins capable of modifying specific target proteins in mammalian cells.[1] PE is consisted of three functional and structural domains as shown by x-ray crystallography.[2] Domain Ia (amino acids 1-252) is responsible for cell recognition, domain II (amino acids 253-364) is involved in the translocation of the toxin to the cytosol and domain III (amino acids 400-613) catalyzes the irreversible ADP-ribosylation of elongation factor 2 (EF-2), leading to inhibition of protein synthesis and consequently to cell death. The exact function of domain Ib (amino acids 345-404) has not been defined.[3-7] In recent years, studies on the molecular mechanism of the PE cytotoxicity showed an induction of apoptosis by activation of caspase-3 and caspase-8. It has shown that high cytotoxicity of PE can be exploited for construction of immunotoxins, a new class of immunotherapeutic agents against cancer.[8-9] In these molecules, the active enzymatic domain of PE is specifically directed to tumor specific antigens. For immunotoxin design, a truncated form of PE, lacking the receptor binding domain, is conjugated to a ligand which is specific to an antigen on the tumor cell surface. The toxin domain internalizes to the cells cytosol and catalytically kills tumor cells.[10-14] RFB4 (dsFv)-PE38 is an example for a disulfide-stablized recombinant immunotoxin, also known as BL22, that is constructed by fusion of Fv domain of anti-CD22 monoclonal antibody RFB4 to PE38 (Li 1989, Pastan 2007). It shows specific cytotoxicity in patients with a variety of B-cell malignancies.[15] The objective of the present study was to clone and express the truncated PE in *E. coli* for the production of recombinant toxin.

Materials and Methods

Bacterial growth and isolation of DNA

Pseudomonas aeruginosa strain PAO1 (ATCC 47085) was cultured in Luria-Bertani (LB) medium at 37˚C with shaking (250 rpm) for overnight. DNA was extracted by proteinase K Phenol/chloroform method as described previously (ref).

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PCR amplification and cloning of truncated PE

1 µl of bacterial DNA was used for a polymerase chain reaction (PCR) with primers PE-F: 5´-ACCCGTCATCGCCAGCC-3´ and PE-R 5´-TTACTTCAGGTCCTCGCGCG -3´ corresponding to the nucleotides 1637-1653 and 2643-2662 of PE gene, respectively. PCR amplification was performed in a total volume of 25 µl containing 0.2 µl of Taq DNA polymerase (Fermentas Inc.), 0.2 mM of dNTPs (dATP, dCTP, dTTP and dGTP), 2 mM $MgCl_2$ and 10 pM of each primer. The PCR program was initial denaturation at 94°C for 4 minutes followed by 35 cycle of denaturation at 94˚C for 1 min, annealing at 58˚C for 1 min, extension at 72˚C for 30 s with a final extension at 72˚C for 5 min. The PCR product was electrophoresed on 1% agarose gel and visualized on UV light after staining with etidium bromide.

Cloning and sequencing of PE fragment

The PCR product was purified with a PCR purification kit (Qiagen) according to the manufacturer's instructions and used for T-A cloning using pGEM-T Easy cloning kit (Promega), yielded the plasmid clone pGEM-PE. The reaction was transformed into an *E. coli* DH5a competent cells and a positive clone was submitted for sequencing. Nucleotide and predicted amino acid sequences were compared to data available by the BLAST search method.

Expression of recombinant Truncated PE

The insert was isolated from pGEM-PE clone by digestion with *NdeI-NotI* restriction enzymes and subcloned into the pET 22b expression vector (Novagen) in frame with a N-terminal six histidine tag, yielding the plasmid subclone pET22b-PE. The construct was transformed into *E. coli* Bl21 strain, cultured at 37 °C in LB medium containing 100 µg ml^{-1} ampicillin to an absorbance at 600 nm (A_{600}) of 1. Protein expression was induced by adding isopropyl--D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and analyzed by SDS-PAGE.

Purification of recombinant protein

For purification of recombinant protein, 500 ml LB media containing 100 µg ml^{-1}ampicillin was inoculated with 5 ml of an overnight culture of recombinant bacteria and incubated at 37°C with vigorous shaking (150 rpm). Culture were induced at mid-log phase by adding 0.5 mM IPTG and growth was continued for another 6 h. Bacteria was harvested (10000 x g, for 10 min), resuspended in 10 ml lysis buffer A (50 mM NaH_2PO4, pH-8, 300 mM NaCl, 10 mM imidazole, 1mg/ml lysozyme) and disrupted by sonication (five 30 sec pulses interrupted with cooling on ice). The cell lysate was centrifuged at 14000 g for 20 min at 4˚C and supernatant was applied onto a nickel (Ni)-charged affinity column (Qiagen) according to the manufacturer's instructions. The column was washed with 5 mM and 20 mM of imidazole and proteins were eluted with 250 mM imidazole. Each of the fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel (12%) electrophoresis (SDS-PAGE) followed by staining with Coomassie Blue G250. Protein concentration was measured using the Bradford assay.

SDS-PAGE Analysis

Sodium dodecyl sulfate-poly acryl amide gel electrophoresis was performed using the Laemmli buffer system. Prior to electrophoresis, the samples were heated at 100°C for 10 min in dissociating buffer containing 2% SDS and 5% 2-mercaptoethanol. Protein marker used were phosphorylase (97.4 kDa), bovine serum albumin 66.3 kDa), ovalbumin (45.0 kDa), lactate dehydrigenae 35.0 kDa), RE Bsp981 (25.0), beta-lactoglobolin (18.4) and Lysozyme (14.4 kDa).

Western blot analysis

For Western blotting, 15–20 µg of bacterial lysate or 1 µg of purified truncated PE were separated by 15% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, N.H.) as previously described (Towbin et al., 1979). Then the membrane were blocked overnight at 4 ˚C with 5% skim milk in TBS (PBS containing 0.05 % Tween 20) and incubated for 1 h with 1/2000 dilution of anti-exotoxin A antibody (Sigma). After washing, membranes were incubated in a 1/3000 dilution of horse radish peroxidase (HRP) conjugated anti-rabbit immunoglobin (Abcam**)** and reactive bands were detected by DAB substate.

Results

Amplification, cloning and sequencing of truncated PE

PCR amplification of truncated PE gene with designed primers resulted in production of a specific band of 1000 bp which appeared as a specific band in agarose gel electrophoresis (Figure 1). Sequencing of PCR product cloned in pGEMT-easy vector confirmed the identity of product as truncated PE lacking domain I.

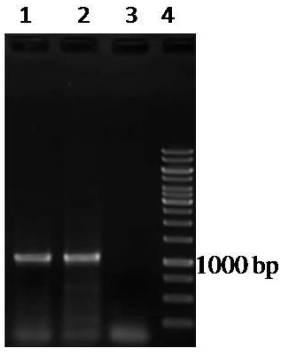

Figure 1. PCR amplification of truncated PE using primers designed for domain 2-3 resulted in a PCR product of about 1000bp. Lane 1 and 2, PE PCR products, lane 3 negative control and lane 4 DNA size marker.

Recombinant expression of truncated PE

The insert was removed from pGEMT-easy vector and subcloned into the pET-22b expression vector through Nde I and Not I restriction enzymes. The expression construct was then transformed into the *E.* coli BL21 cells and subcloning was confirmed by restriction digestion analysis (Figure 2).

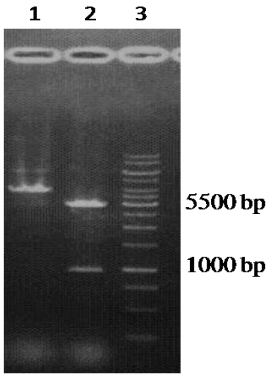

Figure 2. Subcloning of truncated PE coding sequence in pET 22b expression vector. Lane 1, pET22b-PE undigested; Lane 2, pET22b-PE digested with Nde I-Not-I; lane 3, 1kb size marker.

For recombinant expression, the *E. coli* BL21 containing expression construct pET 22b-PE was induced with 1 mM IPTG. Protein expression was analysed by SDS-PAGE and Western blotting. SDS-PAGE analysis revealed high level expression of recombinant PE protein which appeared as a 40 kDa protein in Coomassie blue staining of after induction samples. The level of expression was determined as 30% of total cell protein by densitometry. Western blotting with anti-exotoxin A antibody (Sigma) confirmed the identity of protein and its higher expression with 0.1 mM IPTG (Figure 3).

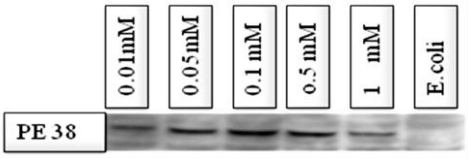

Figure 3. Western blotting analysis of expression level in the presence of different concentration of IPTG. High level expression of soluble recombinant PE38 was obtained in the presence of 0.1 mM concentration of IPTG.

Purification of recombinant PE

Purification of the recombinant product was achieved by Ni-NTA affinity chromatography. The soluble fraction of cell sonicate was passed through Ni-NTA affinity column and eluted with 250 mM imidazol after several washing steps of column. A band corresponding to a 40 kDa recombinant PE was appeared in the SDS-PAGE gels and Western blotting (Figures 4, 5). In this study, about 1.5 mg/ml of recombinant protein was purified by the Ni-affinity chromatography.

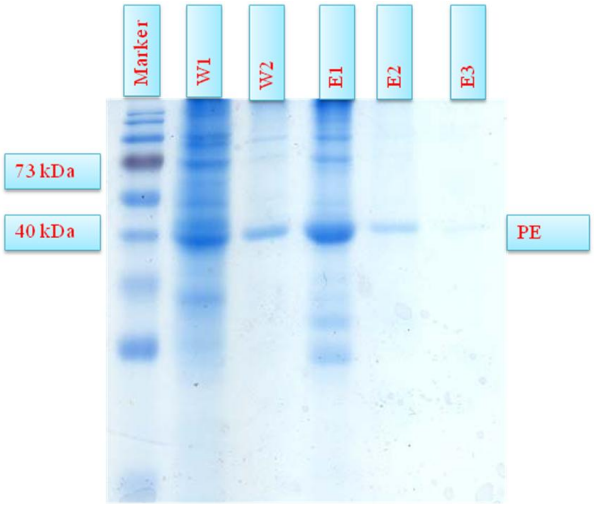

Figure 4. SDS PAGE analysis of different fractions of purification. Most of the protein was eluted with 250 mM concentration of imidazol at E1 (Elution1) fraction.

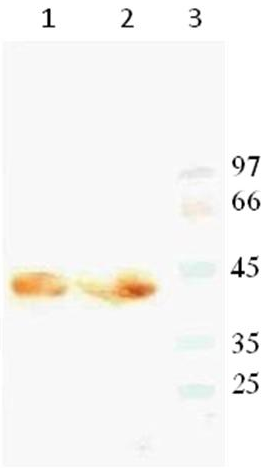

Figure 5. Immunoblotting of purified recombinant PE with anti exotoxin A antibody. Lane 1, elution 1; Lane 2, elution 2.

Discussion

Surgery, radiation and chemotherapy are three main strategies currently used for treatment of cancers, however these procedures affect both normal and cancerous cells resulting in severe side effects. Recent advances in diagnosis of tumor-associated antigens and molecular pathways involved in cancer development lead to design of novel strategies for cancer therapy. One of these strategies is targeting of tumoral cells by immunotoxins. Immunotoxins are created by conjugating a toxin to an antibody or ligand that target specifically tumoral cells. PE is one of the most potent bacterial toxins from *Pseudomonas aeruginosa* that acts by inhibition of protein synthesis via ADP-ribosylation of eEF-2. A large number of PE-based immunotoxins have been developed against tumoral antigens that are specific to or overexpressed in cancers and are in preclinical and clinical trials. These studies indicated that PE and its derivatives are efficient

molecule could be used in cancer therapy. Thus, production of recombinant truncated PE would be useful for the construction of immunotoxins.

In the present study *Pseudomonas aeruginosa* PAO1 that is a toxigenic strain was used for DNA extraction and PCR amplification of PE gene. Domain II through domain III was amplified by PCR using specific primers and subcloned into the pET-22b expression vector under signal sequence of Pel-B. Expression by *E.* coli BL21 and pET-22b expression vector leads to high level production of recombinant PE, which purified by Ni-NTA affinity chromatography. These findings are consistent with previous report on expression of PE38, a derivative of PE.[16] Expression under pelB signal peptide resulted in accumulation of soluble recombinant protein in periplasmic space that is in line with previous reports about efficiency of Pel-B signal sequence.[17] Taken together, this study provides a suitable expression system and a rapid purification method for large scale production of recombinant PE for further use in immunotoxin development studies.

Acknowledgments

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