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Molecular cloning, Expression and purification of Anthrax toxin from *Bacillus anthracis*

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Bacillus Anthracis is the causative agent of anthrax. The major virulence factors are a poly-D glutamic acid capsule and three-protein component exotoxin, which is collectively known as anthrax toxin, protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa), and edema factor (EF, 89 kDa). These three proteins individually have no known toxic activities, but in combination with PA form two toxins (lethal toxin and edema toxin), causing different pathogenic responses in animals and cultured cells. However, it remains to be elucidated for pathogenic mechanism of anthrax toxin. In this study, we constructed toxin component in bacterial overexpression system and purified the native toxin from *Bacillus anthracis delta sterne* F32 using FPLC system. Recombinant toxin showed high homogeneity and rapid purification processes. Also, this recombinant toxin was comparable to *B. anthracis* native toxin in terms of cytotoxic effects on cultured cell lines.

Key words: Anthrax toxin, lethal factor, Molecular cloning, Purification

INTRODUCTION

Anthrax is a zoonotic disease whose etiologic agent is a gram-positive sporulating bacterium, *Bacillus anthracis*. Human beings acquire it via infected animals or contaminated animal products. Virulence of the bacteria is due to two major antigens, antiphagocytic capsular antigen, which is unique among bacterial capsules consisting of poly-D-glutamic acid and tripartite anthrax toxin. Anthrax toxin has three components: protective antigen(PA; 83kDa), lethal factor(LF; 90kDa), and edema factor(EF; 89kDa), of which no single component is toxic but combination of PA and either of LF of EF leads to pathogenesis in laboratory animals [1]. Anthrax toxin, like other bacterial toxins, fits the A-B model of classification of toxins, where B (PA in this case) is the binding moiety, which binds to the cell surface receptors, and LF/EF are alternate catalytic A moieties. PA binds to cell surface receptors, where it is cleaved by furin-like cellular proteases generating a cell-bound, 63kDa protein(PA63). This cleavage of the 20-kDa amino terminal fragment exposes a high affinity binding site on PA63, to which EF or LF binds, which is subsequently internalized by receptor-mediated endocytosis into the lumen of acidic intracellular compartments, the endosomes. EF is a calcium/calmodulin-dependent adenylate cyclase, which increases the intracellular cAMP levels, thereby causing edema. Lethal toxin as the name suggests is lethal for several species. Mouse peritoneal macrophages and macrophage-like cell lines such as J774A.1, RAW 264.7, etc., are sensitive to anthrax lethal toxin. Recent reports indicate that LF acts as an endopeptidase. It cleaves the amino terminus of mitogen-activated protein kinase kinases 1 (MAPKK1), which contain the MAPK binding site, thus prevents the association of MAPKK1 with its substrate and inhibits the MAPK signal transduction pathway. However, the exact mechanism of cell death is not yet established. Although the genes for all three-protein exotoxins have been cloned and sequenced, recombinant production of all three-protein exotoxins was not successful up to date. In this study, we attempted to construct the toxin components with high-level expression and rapid purification system in *E.coli.*

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MATERIALS AND METHODS

Bacteria Strains and Materials. The *E. coli* strain, *DH5α*, and BL21(DE3), were used as a plasmid amplification strain and an expression host strain, respectively. Cell culture media (Bacto-tryptone, Bacto-yeast extract) were purchased from Difco. Restriction enzyme, *Pfu* DNA polymerase, and T4 DNA ligase were from Promega. DNA prep

kit and Gel extraction kit were purchased from Q-Biogene. The plasmid coding to GST-MEK$^{(-)}$ was kindly provided by Dr. Kun-Liang Guan (Department of Biochemistry, University of Michigan, U.S.A). The other chemicals used here including isopropyl-1-thiogalactopyranoside were form SIGMA.

Molecular Cloning of anthrax toxin. Total genomic DNA of *Bacillus anthracis* delta F32 was isolated by the method described elsewhere. 100 ng of purified total genomic DNA was used as a template for the polymerase chain reaction (PCR). The sequences of specific primer set for LF were 5'-aatctagacgcgggcggtcatggtgatgta, 5'-aagtcgacttatgagttaataatgaactt with Xba I and Sal I, respectively. PCR products were purified by the extraction with phenol:chloroform followed by ethanol precipitation. PCR fragment of LF and pGEX-KG, an expression vector, were digested with Xho I and Sal I and were subjected to ligate by T4 DNA ligase. This product was transformated to *E. coli,* DH5α, and transformants were selected on LB plate containing appropriated antibiotics, ampicillin. Colonies were selected and screened to isolate the plasmid containing the gene coding lethal factor. Constructed DNA was further identified by restriction enzyme mapping with the fragment from the digestion of LF gene specific restriction enzyme and by DNA sequencing.

Overexpression of Recombinant Protein. The plasmid containing wild type LF was transformated into expression host, BL21(DE3), which has relatively low protease activity, and the single colony of these cells was grown in LB medium containing 50μg/ml ampicillin at 37℃ for overnight. Appropriate volume of subculture was transferred to the large volume of culture media (1 L) and further incubated until $O.D_{600}$ = 0.8. Overexpression of target protein was induced by adding isopropyl-1-thiogalactopyranoside to a final concentration of 1 mM and cells were harvested at 5 h post-induction.

Purification of Recombinant Protein. Cells were harvested by centrifugation at 5,000 rpm at 4℃ for 10 min. All the subsequent procedures were performed on ice. Pellets were resuspended in the PBST buffer containing 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.4, 2 mM EDTA, 1% Triton X-100, and 0.1% BME and incubated on ice for 10 min, followed by lysis with ultrasonication. Resultant cell lysates were directly incubated with glutathione (GSH)-sepharose 6B resin that was pre-equilibrated with PBST buffer. This batch type method provided the rapid and convenient purification tools without any concentration steps before binding with resin. 1 ml of 50% slurry of GSH-sepharose resin was added to cell extracts and incubated at 4℃ for 2 hours with mild shaking. After incubation, target protein bound to the resin was separated from the total cell extracts by short spin down and vigorously washed with enough volume of PBST containing 500 mM NaCl several times. Recombinant LF was eluted by a step gradient with 20 mM glutathione buffer (pH 8.0) at 4℃ for at least 4 hours and dialysed against the buffer containing 20 mM Tris, pH 8.0, 2 mM EDTA, and 10% glycerol. Purified proteins showed high homogeneity from the single band on 10 % SDS/PAGE analysis. The concentration of proteins was determined by Bradford reagent according to manufacture's direction.

RESULTS AND DISCUSSION

1. Purification of native toxin from *B. anthracis.*

In order to purify the native toxin, *B. anthracis delta sterne* F32, pXO1^{+} and pXO2^{-}, were grown in the complex medium without alanine (modified RM) until A580=2.0-2.5. Since anthrax toxins were exotoxin, culture medium were harvested and then subjected to purify as following procedure (Fig. 1 Scheme).

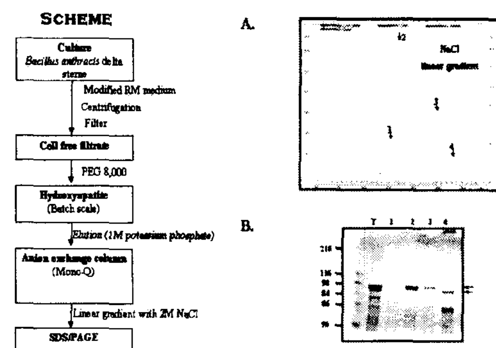

Figure 1. ***Purification of native toxin***. A. 1mg of Eluents from hydroxyapatite were subjected to load on Mono-Q anion exchange column in FPLC system. Samples were eluted by a linear salt gradient with 2M NaCl. B. Each Mono-Q column elution fraction was dialyzed and concentrated by ultrafiltration with Centricon. Pooled fractions were analyzed by 8% SDS/PAGE. (T, crude eluents from hydroxyapatite; 1, 2, 3, 4, fraction from Mono-Q column.)

2. Construction of recombinant anthrax toxin.

The genes for all three-protein components are present on a single 185-kb plasmid, pXO1, while another plasmid, pXO2, contains the genes responsible for poly-D-glutamic acid capsule synthesis. First, we isolate the pXO1 and the genes coding to each toxin were amplified by polymerase chain reaction (PCR) using each gene specific primer containing appropriated linker. PCR fragments were isolated and purified with phenol:chloroform extraction, followed by ethanol precipitation. Genes were subcloned to bacterial overexpression vector and cloned genes were screened and identified by restriction enzyme mapping and DNA sequencing (Fig. 2)

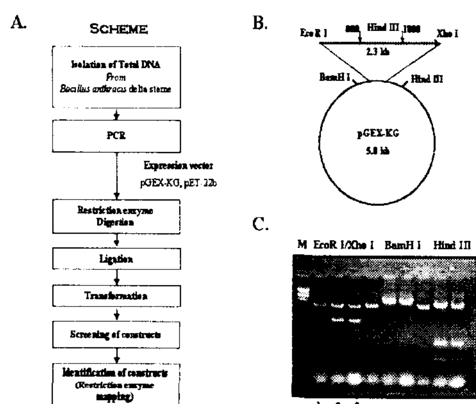

Figure 2. ***Construction of recombinant toxins.*** A. Overall scheme of construction, B. Construction map of recombinant toxin in bacterial overexpression system. Representative diagram is a construct of edema factor, C. Constructed toxin was identified by restriction enzyme mapping. (1. Empty vector; 2,3. Constructed colony # 1, 2; M. lamda Hind III, 24, 9, 6, 4, 2,3, 2.0, 0.56 kb), Expected fragment : EcoR I/ Xho I – 2.3 kb (insert), BamH I – 7.3 kb, Hind III – 1.0 kb, 0.5 kb, 5.8 kb)

3. Overexpression of recombinant toxin.

The plasmid containing anthrax toxin (Representative data was based on lethal factor) was introduced to *E. coli* expression host, BL21(DE3), which showed relatively low cellular protease activity. Cells were grown in LB medium containing 50μg/ml ampicillin and appropriate volume of subculture was transferred to the large culture scale (1L). When cell growth reached to $O.D_{600}$ = 0.8, recombinant toxin was induced to overexpress by adding IPTG with final concentration to 1 mM at 30℃. To set up the optimal overexpression condition, we saved the portion of induced cells at the indicated time period and then cells were completely lysed in the PBST buffer containing 1% SDS. As shown in Fig. 3B, protein expression was significantly increased after 2 hours and maximized at 5 hours post-induction. After 5 hours post-induction, cells were harvested by centrifugation and resuspended in PBST buffer (pH 7.4) as described in *Materials and Method.* Cells were lysed by sonication and resultant lysates was subjected to affinity purification. Since LF is a protease, rapid purification steps should be needed. To set the purification method, we firstly tested classical column chromatography in *Econo system.* 1mg of total protein was load on affinity column, glutathion-sepharose 6B, which was pre-equilibrated with PBST buffer and washed by 10-bed volume of PBST buffer in which the concentration of NaCl was increased to 150mM. Recombinant LF was eluted by linear gradient of glutathion (pH 8.0) to 15 mM. Target protein was eluted in narrow fraction of the glutathion in the range of 7-8.5 mM and these fractions showed high homogeneity from the clear single band on SDS/PAGE (data not shown). In the practical purification process, we performed a stepwise gradient with glutathione.

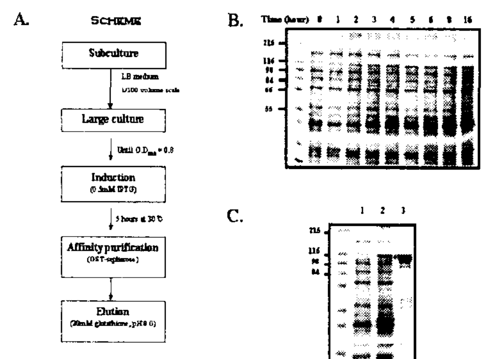

Figure 3. ***Overexpression of recombinant toxin.*** B. Identification of induction. Expression level of target protein was monitored with IPTG induction at 0.5mM for indicated time period. Protein expression was significantly increased after 2 hours and sustained for 16 hours. C. Overexpressed proteins were purified by affinity resin, GST-sepharose, with a step gradient. (1. Total cell lysates before induction, 2. Total cell lysates after induction, 3. Purified toxin eluted by 7-8.5mM glutathione)

Reference

1. Leppla, S. H. (1988) Production and purification of anthrax toxin. Methods in Enzymology. 165 103-116