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Secretory expression and efficient purification of recombinant anthrax toxin lethal factor with full biological activity in *E. coli*

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ABSTRACT

Lethal factor (LF), a virulence factor of *Bacillus anthracis*, plays key roles in anthrax pathogenesis and host-pathogen interactions. The detailed mechanisms by which LF contributes to infection are still under investigation. While these studies require pure, homogeneous and reliable LF preparations, most methods reported for production of recombinant LF (rLF) in *B. anthracis* or *Escherichia coli* either are complicated or add extra residues to the protein. In this work, we modified our previous method by codon optimization and chromatograph workflow refinement and developed an improved strategy for efficient production of rLF from the periplasm of *E. coli*. We were able to obtain fully functional rLF with a purity above 95% and with a considerable yield of 5 mg/L. The preparation was characterized by SDS–PAGE, Western blot, and N-terminal sequencing, and the activity was validated by intoxication of macrophages and Fischer 344 rats. Our final product is suitable for most research involving drug development and mechanism analysis of anthrax pathogenesis.

Introduction

Lethal factor (LF)[2] is a secreted virulence factor of *Bacillus anthracis*, and forms the lethal toxin (LT) with another secreted protein, protective antigen (PA) [1]. After entering the cytosol through pores of PA forming on the endosome membrane, LF cleaves the N-terminus of a number of mitogen activated protein kinase kinases (MAPKKs, or MEKs) as a zinc metalloproteinase [2–3]. This disrupts the ERK1/2, JNK/SAPK, and p38 signaling pathways pivotal for cellular functions including proliferation, cell cycle regulation, immune modulation, and survival under pressure [4–5]. Ultimately, this LF-mediated disruption of cellular signaling results in macrophage lysis [6–7], vascular barrier disruption [8–12], pleural edema [13], and rapid death (40 min) in Fischer 344 rats [14]. In this manner, LF plays a critical role in the pathogenesis of anthrax by helping the bacteria to manipulate the host immune system, proliferate, and kill the host [10]. Nevertheless, the detailed mechanism by which LF contributes to infection is still under investigation. Such studies require pure, homogeneous and reliable lethal factor preparations in order to obtain accurate and reproducible results.

Many attempts have been made to produce native or recombinant LF (rLF) in *B. anthracis* or *Escherichia coli* [15–20]. However, no promising solution has been achieved. A main troublesome factor is the extra residues added to the ends of the peptide. Other issues complicating rLF preparations are biosafety considerations, inefficient or laborious purification procedure.

Previously, we have demonstrated that rLF with native termini can be obtained with a periplasmic expression strategy in *E. coli* [21]. This method includes cloning the LF coding sequence downstream of the *OmpA* signal sequence of the secretory expression plasmid, pAS22, and periplasm extraction followed by three chromatography steps. However, there are still limitations with this strategy. Specifically, the expression level is low and the purification is inefficient, resulting in poor yield (about 3 mg/L) and an unsatisfactory final purity (about 90%).

In this study, we modified our previous method by codon optimization and chromatograph workflow refinement. As a result, the yield and purity were improved significantly. Typically, 5 mg of rLF protein with over 95% purity was obtained from one liter of *E. coli* culture. The characterization and biological activity of rLF were validated in *vitro* and in *vivo* respectively, which demonstrated that the product is suitable for most anthrax toxin research.

Material and methods

Bacteria strains and reagents

The *E. coli* strain *DH5α* (Tiangen Biotech, Beiging) was used as the host for both cloning and expression. Enzymes used for subcloning were purchased from New England Biolabs. A rabbit polyclonal antibody to LF was prepared previously in our laboratory. A

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[1] These authors contributed equally to this work.
[2] *Abbreviations used:* LF, Lethal factor; LT, lethal toxin; PA, protective antigen; rLF, recombinant LF.

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commercially available rLF used as control was purchased from List Biological Laboratories. All other chemicals and reagents, unless otherwise noted, were from Sigma.

Mutation for truncated rLF and expression analysis

The LF expression plasmid, pAS22-LF, was previously constructed and preserved in our lab [21]. Briefly, the pAS22 vector was constructed based on the pASK84 vector [22] and contains a lac promoter, an *OmpA* signal sequence, and multiple cloning sites. The coding sequence of the mature peptide of LF was amplified from the *B. anthracis* A16R strain (GenBank accession No. AY997300.1), and was ligated into the pAS22 between the 5′ StuI site and 3′ SalI site after the *OmpA* signal sequence. Inverse PCR was applied for the construction of the expression vector for truncated rLF. Primer Trf (GAGCTCTAGAGTCGACCTGCAGGC) was used as the forward primer and primers Trr2 (CCCTAATGCTTTATTCCATT CCTGATTTATAT) and Trr4 (CTCTTTTTCTTTTTCAGATAAAGGATTAC-TACTATCC) were used as reverse primers to obtain expression vectors which lacked 201 and 402 amino acid residues at the C-terminus of LF, respectively. Reconstructed plasmids were transformed into *DH5α* competent cells.

To compare the expression levels of rLF and truncated rLF, small batch trials were performed. Briefly, a single colony for each mutant was grown in 3 mL LB containing 100 μg/mL ampicillin overnight at 37 °C, and 50 μl of the subculture was transformed into 5 mL LB and incubated further at 37 °C until the OD_{600} reached 0.6–0.8. IPTG was added into the medium at a final concentration of 0.4 mM, and the culture was further incubated at 28 °C for about 5 h. Then the cells were harvested by centrifugation with 8000*g* for 10 min at 4 °C. All the subsequent procedures were performed on ice. The pellets were washed twice with 2 mL of PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 2 mM KH_2PO4, pH 7.4), resuspended in 500 μl PBS buffer, and disrupted by an ultrasonic processor. The cell lysate was analyzed by 12% SDS–PAGE.

Codon optimization and expression vector construction

For codon optimization, a net tool (www.jcat.de) [23] was used according to instructions, and the codon adaptiveness of *E. coli* K-12 strain was applied. Two segments in the gene sequence with extremely rare codons (931–1350, denoted as F1, and 1540-end, denoted as F2) were optimized and synthesized (SUNBIO technology Inc., Beijing, China; sequences attached in the Supplementary section). For F1, the inverse PCR product was amplified from the pAS22-LF plasmid by primers VecF (ACCTTGTACAATAAAATTTATTT GTATG) and VecC (TCCTTCTTCAGATAAAGAATCGC) and the 5′ phosphorylated fragment product was amplified from the plasmid containing the synthetic fragment F1 by primer pairs FragF (CGTGGTCTGCTGAAAAAACTGC) and FragR (AGAACCGATAGACTGG TGCAGC). The two amplification products were ligated to replace the original sequence in pAS22-LF, and the new recombinant plasmid was denoted as pAS22-F1. For F2, taking advantage of the internal unique restricted enzyme sites (StuI at the 5′ and SacI at the 3′), the synthetic segment was ligated into the plasmid pAS22-LF and pAS22-F1, and the new recombinants were denoted as pAS22-F2, and pAS22-F1+F2, respectively. All plasmids were validated by sequencing and were transformed into *DH5α* competent cells.

Expression and purification

The starting culture was prepared by stabbing the glycerol stock into 3 mL LB with 100 μg/mL ampicillin and then incubating overnight at 37 °C. A 1:20 scale-up was initially followed by a 1:100 scale-up after a 4 h incubation at 37 °C, 220 rpm. For production, a 5 L flask with 1 L culture was used. When OD_{600} reached 0.6, the culture was induced with 0.4 mM IPTG for 5 h at 28 °C, 220 rpm. Bacteria were harvested by centrifugation at 8000*g* for 10 min at 4 °C. The pellet from the 1 L culture was resuspended with 50 mL 20% sucrose, 20 mM Tris pH 8.0, and 1 mM EDTA. After incubation on ice with gentle stirring for 5 min, the suspension was centrifuged at 22,000*g* for 10 min. The supernatant of this hyper-osmotic step was kept, and the pellet was resuspended with 50 mL 0.1 mM $MgCl_2$. After incubation on ice with gentle stirring for 10 min, the suspension was centrifuged at 22,000*g* for 10 min. The supernatant of this step was mixed with the supernatant of the hyper-osmotic step as the periplasm extraction sample. Samples were first loaded on a CHT™ ceramic hydroxyapatite column (Bio-rad, USA) equilibrated with 0.005 M phosphate sodium at pH 7.2, washed with 0.08 M phosphate sodium at pH 7.2, and eluted with a 0.08 M to 0.18 M phosphate sodium linear gradient at pH 7.2. Following analysis with SDS–PAGE, selected fractions were concentrated and the buffer was changed to 20 mM Tris at pH 8.0 by ultrafiltration with an Amicon® Ultra-15 10 K centrifugal filter device (Millipore, USA), and then loaded onto a prepacked source 30Q column (GE healthcare, USA). Samples were then eluted with a 0.125 M to 0.2 M NaCl gradient, supplemented with 20 mM Tris at pH 8.0. Following analysis with SDS–PAGE, selected fractions were concentrated and the buffer was changed to PBS by ultrafiltration with the same device.

SDS–PAGE and Western blot analysis

SDS–PAGE (12%) was applied and gels were stained with Coomassie Blue and analyzed with Image J (http:// imagej.nih.gov/ij, NIH, USA) to determine purity. Western blot was then employed to detect recombinant proteins. Briefly, samples in the gels were electrotransferred to an Amersham™ Hybond™-ECL 0.45 μm membrane (GE healthcare, USA). The blot was labeled with anti-LF pAb and an HRP labeled goat anti-rabbit secondary antibody (Sigma, USA), and developed with the Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, USA).

Protein N-terminal sequencing

The protein samples (two different batches of preparations) with purity above 95% were sequenced by the National Center of Biomedical Analysis (Beijing, China) using an ABI *Procise 491* (*ABI, USA*) *protein sequencer*.

Cytotoxicity assay

Mouse macrophage J774A.1 cells were plated at 3×10^5 cells/well in 96-well plates and cultured for 24 h before treatment. A dilution series of the LF preparations, combined with PA at 1 μg/mL, was added to the cells to a final volume of 100 μl/well and incubated at 37 °C. Cell viability was assayed 4 h after treatment by replacing the medium with 100 μl of medium (MEM plus 2% FBS) containing 1 mg/mL MTT (Invitrogen, USA). After 1 h of incubation at 37 °C, the medium was removed and the blue pigment produced by the viable cells was dissolved in 50 μl/well of 0.5% (w/v) SDS and 25 mM hydrochloric acid in 90% (v/v) isopropanol. The plates were then vortexed, and oxidized MTT was measured as absorbance at 570 nm using a Model 550 microplate reader (Bio-Rad, USA). Cell viability was calculated as a percentage using the equation $(OD_{sample} - OD_{death\ control})/(OD_{live\ control} - OD_{death\ control})$, where "live control" wells contained 1 μg/mL PA alone and "death control" wells contained both 1 μg/mL PA and 1 μg/mL LF. EC50 values were determined by nonlinear regression sigmoidal dose–response analysis with variable slopes (Prism, version 4.0,