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Efficient production and characterization of *Bacillus anthracis* lethal factor and a novel inactive mutant rLFm-Y236F

Sha Cao [a,b], Ziduo Liu [a], Aizhen Guo [a,b,*], Yan Li [a,b], Chengxian Zhang [a,b], Wu Gaobing [a], Feng Chunfang [a], Yadi Tan [a], Huanchun Chen [a,b,*]

[a] *National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China*
[b] *Provincial Key Laboratory of Preventive Veterinary Medicine, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, PR China*

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Abstract

Lethal factor (LF) is a 90 kDa zinc metalloprotease that plays an important role in the virulence of anthrax. Recombinant LF (rLF) is an effective tool to study anthrax pathogenesis and treatment. In this study, the LF gene was cloned into the *Escherichia coli* expression vector pGEX-6P-1 and expressed as a GST fusion protein (GST-rLF) in *E. coli* BL21-codonPlus (DE3)-RIL cells with 0.2 mM IPTG induction at 28 °C. The GST-rLF protein was purified and the GST-tag was then cleaved in a single step by combining both GST-affinity column and treatment with 3C protease. This procedure yielded 5 mg of rLF protein per liter of culture. The purified rLF was functional as confirmed by cytotoxicity assay in RAW264.7 cells and Western blot assay. Furthermore, the rLF could induce strong immune response in BALB/c mice and the presence of a specific antiserum could neutralize the cytotoxicity of rLF *in vitro*. In addition, a novel inactive mutant (rLFm-Y236F) was obtained. Compared to the wild-type rLF, an increase by 3700 folds of the purified rLFm-Y236F was needed to achieve a similar level of cytotoxicity of the wild-type rLF. This mutant might be of significance in the study of anthrax pathogenesis and treatment.

Keywords: *Bacillus anthracis*; Lethal factor; Anthrax; Lethal toxin; Protein purification

Anthrax is a zoonotic contagious fatal disease caused by *Bacillus anthracis* (*B. anthracis*). It is primarily a disease of herbivores, but all mammals, including humans, are susceptible hosts. In 2001, the bioterrorists intentionally released anthrax in the United States and resulted in 11 cases of inhalational disease with an attendant mortality rate of 45% [1].

Bacillus anthracis is a rod-shaped, gram-positive, and spore-forming bacterium. The infection is initiated by the entry of spores into the host body through a minor abrasion, an insect bite, or by eating contaminated meat or inhaling airborne spores [2,3]. Therefore the manipulation of the bacteria needs biosafety level 3 facilities.

The main virulence factors of *B. anthrasis* include poly-D-glutamic acid capsule and anthrax lethal toxin (LeTx)[1] [3]. LeTx plays a key role in anthrax pathogenesis both in the early stages of the disease and throughout the disease progression, while the poly-D-glutamic capsule protects *B. anthrasis* from being killed by macrophages [3,4]. LeTx is a binary AB toxin composed of three secreted proteins protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa), and edema toxin (EF, 89 kDa). PA, the B part of toxin, plus A part LF calls lethal toxin, while PA when combined with another A part EF forms edema toxin [2,5,6]. Because LeTx treatments could reproduce most symptoms of *B. anthracis* infection, LeTx has been utilized

* Corresponding authors. Fax: +86 27 87281795.
E-mail addresses: aizhen@mail.hzau.edu.cn (A. Guo), chenhch@mail.hzau.edu.cn (H. Chen).

[1] *Abbreviations used:* LeTx, anthrax lethal toxin; PA, protective antigen; LF, lethal factor; EF, edema toxin; rLF, recombinant LF; PBS, phosphate-buffered saline.

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in studying anthrax pathogenesis [7,8] and treatment [1]. Since antimicrobials only target replicating organisms, thus leaving bacterial toxins to cause unchecked physiological derangements in the host, novel approaches that target the cytotoxic effects of anthrax exotoxins are pursued including the development of new chemicals [1,9], use of antibodies [10], and toxin mutants [11–13].

LF is a Zn^{2+}-endopeptidase specific for the MAPK-kinase family of proteins [6] and a key virulence factor for *B. anthracis* since mouse death was seen in the absence of edema factor, but not in the absence of lethal factor [14]. It causes cell death by preventing the association of MAPKK1 with its substrate and inhibits the MAPK signal transduction pathways including ERK (extracellular signal-regulated kinase), p38 MAPK and JNK (c-Jun N-terminal kinase) pathways [5,15]. Therefore, inhibition of this proteolytic-based LF toxemia could eventually provide a therapeutic value in combination with an antibiotic treatment during and immediately after an active anthrax infection [16]. As mentioned previously, biologically active LF might be purified from *B. anthracis* culture [17]. However, the hazardous *B. anthracis* restricts the ordinary laboratories to perform such purification. Although LF has been expressed in *E. coli* [2,17–19], the conventional purification strategies involve complicate purification procedure (e.g., gel filtration, Mono Q FPLC, anion exchange) and tedious changes of buffers and are not suitable for a protease like LT which is easily degraded. Thus a simple and straightforward method to obtain high quality recombinant LF (rLF) or its mutants is needed. To this end, several strategies have been utilized, such as fusion of the LF gene to the leader sequence of outer membrane protein OmpA so that the rLF can be translocated into the periplasm and its degradation was decreased, or fusion of LF to a His Tag to facilitate protein purification by one-step nickel affinity column [18–20]. However, it is necessary for these methods to remove unbeneficial reagents present in buffers by dialysis or cleave the fused tags by additional steps. In this study, we expressed rLF as a fusion protein with GST-tag (GST-rLF) in *E. coli* and the GST-tag was cleaved through a single affinity purification step which used only phosphate-buffered saline (PBS) buffer in the whole purification procedure. At the same time, a novel rLF mutant (rLFm-Y236F) was obtained. The cytotoxicity analysis demonstrated that the purified rLF was fully functional, while the rLFm-Y236F lost its activity. The purification method developed in this study combined the new mutant might provide useful tools for the research in anthrax-mediated pathogenesis and anthrax therapy.

Materials and methods

Chemicals and reagents

The 3C protease ("PreScission") fused with GST was used to cleave glutathione *S*-transferase (GST) tag from the fused protein GST-rLF and kindly offered by Prof. Liu Ziduo in this Lab. GSH-Sepharose 4 Fast Flow was purchased from Amersham Pharmacia Biotech. All other chemicals and reagents except otherwise noted in this study were purchased from Sigma.

Strains and cells

Escherichia coli DH5α was used for general cloning and maintenance of plasmids. BL21-CodonPlus (DE3)-RIL strain was used as a host for protein production. RAW264.7, a murine macrophage cell line (CTCC) was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum.

Plasmid construction and protein expression

The entire LF gene (GenBank Accession No. AF065404), except for the portion that encodes the signal sequence, was amplified by PCR from noncapsulogenic Sterne strain of *B. anthracis* (Tecon Biotechnology Limited Company) by using following two primers: sense primer, 5′-GTT CAG GAT CCG CGG GCG GTC ATG GTG AT-3′ (the *Bam*HI site is underlined); antisense primer, 5′-GCT GAG CGG CCG CTC ATT ATG AGT TAA TAA TGA ACT TAA TC-3′ (the *Not*I site is underlined). The LF gene was cloned into pGEX-6P-1 by using *Bam*HI and *Not*I sites to obtain pGEX-6P-1-LF. The accuracy of cloned sequences was confirmed by sequencing at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

To optimize LF expression, BL21 (DE3)-RIL competent cells were transformed with pGEX-6P-1-LF or pGEX-6P-1-LFm. A single colony was grown in 5 ml LB medium containing 100 μg/ml ampicillin at 37 °C. When OD_{600} reached 0.8, IPTG was added into the medium at final concentrations of 0.2 mM, 0.4 mM, and 0.8 mM, respectively. The culture was further incubated at either 28 °C or 37 °C. The rLF or the mutant rLFm expression was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) after induction for 2, 4, 6, and 8 h.

For small batch trial, a single colony of the cells was grown in 10 ml LB medium containing 100 μg/ml ampicillin overnight at 37 °C, and the 10 ml subculture was transformed into 1 L LB and incubated further at 37 °C. After an induction at the optimized condition, cells were harvested by centrifugation with 4000*g* for 10 min at 4 °C. All the subsequent procedures were performed on ice. The pellets were washed with 200 ml of PBS buffer (140.0 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), resuspended in 50 ml PBS buffer, and disrupted by using French cell press. The cell lysate was centrifuged at 10,000*g* for 30 min and the supernatant was saved for GST-free affinity purification.

The Gutathione (GSH)-Sepharose column (bed volume, 1 ml) was pre-equilibrated with 50 ml PBS buffer. The clear supernatant was directly packed into the GSH-Sepharose column with an initial elute speed of about 1 ml/min. The