Expression and Purification of the Recombinant Lethal Factor of *Bacillus anthracis*

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The structural gene for the 90-kDa lethal factor (LF) isolated from *Bacillus anthracis* was expressed as a fusion protein with six histidine residues in *Escherichia coli*. Expression of LF in *E. coli* under the transcriptional regulation of the T5 promoter yielded a soluble cytosolic protein with an apparent molecular mass of 90 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Recombinant LF reacted with anti-LF antibodies. The protein was purified to homogeneity by nickel nitrilotriacetic acid affinity chromatography and gel filtration on a Sephacryl S-200 column followed by anion exchange on a fast-performance liquid chromatograph with a Resource-Q column. The yield of purified LF from this procedure was 1.5 mg/liter. In solution, trypsin cleaved protective antigen bound to native and recombinant LF with comparable affinities. In macrophage lysis assays, native and recombinant LF exhibited identical potencies. The results suggest that large amounts of biologically active LF can be purified by this procedure.

The major virulence factors of *Bacillus anthracis*, the causative organism of anthrax, are a poly-D-glutamic acid capsule and a three-component protein exotoxin. The genes coding for the toxin and the enzymes responsible for the capsule production are carried on *B. anthracis* plasmids pXO1 and pXO2, respectively (8, 19). The three proteins of the exotoxin are protective antigen (PA [83 kDa]), lethal factor (LF [90 kDa]), and edema factor (EF [89 kDa]). The toxins follow the A-B model, with the A moiety being the catalytic part and the B moiety being the receptor binding part. PA acts as the B moiety and binds to the cell surface receptor, while LF and EF compete for binding to PA (18, 25). LF and EF are individually nontoxic but in combination with PA form two distinct toxins causing different pathogenic responses in animals and cultured cells (6). In the process of cytotoxicity, PA binds to cell surface receptor and is cleaved at the sequence RKKR^{167} by cell surface proteases such as furin, generating a cell-bound, C-terminal, 63-kDa protein (PA$_{63}$). PA$_{63}$ possesses a binding site to which LF or EF bind with high affinity (11, 28). The complex formed by EF bound to PA$_{63}$ is known as edema toxin, which causes edema when injected into the skin of animals (30). EF has calcium- and calmodulin-dependent adenylate cyclase activity and in combination with PA dramatically increases cyclic AMP levels of cells (13). LF bound to PA$_{63}$ produces lethal toxin, which causes death in experimental animals and lysis of mouse peritoneal macrophages and macrophage-like cell lines such as J774A.1 and RAW264.7 (6, 7, 30). After binding of LF (or EF) to PA$_{63}$, the complex is internalized by receptor-mediated endocytosis (3, 6). Following internalization of LF, there is an increase in macrophage permeability to Na$^+$ and K$^+$, which is followed by hydrolysis of ATP (9). Subsequently, there is influx of Ca^{2+} (3) and leakage of cytoplasmic lactate dehydrogenase, leading to cell death. Protein synthesis is required to express cytotoxicity in J774A.1 cells (4). However, the exact molecular mechanism of cell killing is not yet understood. LF has been proposed to be a zinc-dependent metalloprotease; however, its substrate is not yet known (12).

The genes for PA, LF, and EF have been cloned and sequenced (5, 24, 31). Protective antigen has been expressed and purified from *Bacillus subtilis* as well as from *Escherichia coli* (10, 27, 28). Attempts to express and purify LF from *B. subtilis* were not very successful (15, 16). The LF gene coding for amino acids 1 to 776 (LF$^{1-776}$) and LF$^{1-254}$ has been expressed in *E. coli* with various promoters (1, 2, 23). In these studies, the degree of expression of LF$^{1-254}$ has been obtained to significant levels. However, isolation of full-length LF did not yield significant amounts of LF due to extensive proteolytic degradation in the cytosol (22). Klimpel et al. expressed LF as a fusion protein along with 164 amino acids of PA in *B. anthracis* to enhance the expression of LF (12). The fusion protein required trypsin cleavage to obtain mature LF. To date, culture supernatants of *B. anthracis* have been the major source for the purification of LF (14, 21). Purification of LF from *B. anthracis* requires containment facilities because of the highly infectious nature of the organism. In addition, LF preparations from *B. anthracis* are invariably contaminated with other proteins with either similar charges or molecular masses such as PA, EF (30), and phospholipase C (2a). Currently, no simplified system to obtain full-length homogeneous preparations of LF in appreciable amounts from *E. coli* is available.

In this study, attempts have been made to express the full-length LF as a fusion protein with six histidine residues in *E. coli*. Recombinant LF (rLF) was purified by affinity chromatography, gel filtration, and finally ion-exchange chromatography.

Plasmid construction and expression of LF. Plasmid pLF7, a generous gift from Stephen H. Leppla, National Institute for Dental Research, National Institutes of Health, contains the entire native LF (nLF) gene. Expression vector pQE30 (Qiagen) contains the T5 promoter for high-level expression, a ribosome binding site, and × histidine coding sequences followed by a multiple cloning site. The vector also contains two *lac* operator sequences. Plasmids pLF7 and pQE30 were purified with the DNA purification kit (Qiagen), as described in the manufacturer's manual. The LF gene was amplified by

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PCR with pLF7 (23) as a template and primers that added *Bam*HI and *Sal*I sites to the 5′ and 3′ ends of the PCR product, respectively. Sequences of the forward and reverse oligonucleotides were 5′ GTA CAG GGA TCC GCG GGC GGT 3′ and 5′ GAA AAT TTT TAA TAG TCG ACT TAT GAG 3′, respectively. The amplified PCR product and plasmid pQE30 were digested with restriction enzymes *Bam*HI and *Sal*I. The digested products were separated on a 1% agarose gel. The bands were excised, and the DNA was eluted with the gel extraction kit. The digested PCR product and the vector were ligated overnight at 14°C and transformed into *E. coli* SG13009 (pREP4)-competent cells. Preparation and transformation of competent *E. coli* SG13009 bacteria were performed according to procedures described by Maniatis et al. (26). The transformation mixture was plated on Luria agar plates containing 100 μg of ampicillin per ml and 25 μg of kanamycin per ml. The plates were incubated for 16 h at 37°C. Colonies appearing on the plate were screened for the recombinant plasmid pPG-LF1 by minipreparations of plasmid DNA (26). The desired recombinant plasmid was confirmed by restriction enzyme digestion with *Bam*HI and *Sal*I. For high-level expression of the gene, SG13009(pREP4) cells containing multiple copies of the plasmid pREP4, which carries *lacI*^q gene encoding the *lac* repressor, were used. Multiple copies of pREP4 present in the host cell ensured high levels of *lac* repressor and tight regulation of protein expression. Expression of rLF was established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Cells carrying the plasmid pPG-LF1 were grown and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Periplasm, cytosol, and inclusion bodies were checked for the presence of LF. LF was found to be mainly localized in the cytosol (Fig. 1).

Purification of LF. *E. coli* SG13009(pREP4) carrying the recombinant plasmid pPG-LF1 was grown at 37°C in Luria broth with 100 μg of ampicillin and 25 μg of kanamycin per ml at 250 rpm. When the A_{600} reached 1.0, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. After 5 h of induction, the cells were harvested by centrifugation at 4,000 × g for 20 min. For the purification of protein from 2 liters of culture, the pellet was resuspended in 50 ml of sonication buffer (50 mM Na phosphate [pH 7.8], 300 mM NaCl). Lysozyme (1 mg/ml) was added to the slurry and incubated on ice for 30 min. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM. Cells were sonicated at 4°C (1-min bursts, 1 min of cooling, 200 to 300 W) for five cycles. The lysate was centrifuged at 10,000 × g for 30 min. The supernatant was mixed with 8 ml of a 50% Ni-nitriloacetic acid resin previously equilibrated with sonication buffer. The slurry was packed into a column (5.0 by 1.6 cm) and allowed to settle. The matrix was washed first with sonication buffer followed by wash buffer (50 mM Na phosphate [pH 6.0], 500 mM NaCl, 10% glycerol). The column was washed until the A_{280} of the flowthrough was less than 0.01 (approximately 50 ml). Protein was eluted with a linear gradient of 15 ml each of 0 and 500 mM imidazole chloride in elution buffer (50 mM Na phosphate [pH 7.0], 100 mM NaCl, 10% glycerol). Fractions of 1 ml were collected and analyzed on an SDS-10% PAGE gel. rLF eluted at a gradient of 100 to 250 mM imidazole chloride. Affinity-purified protein possessed full-length rLF, and approximately 40% degraded LF as determined by SDS-PAGE and Western blotting with anti-LF antibodies (Fig. 2). The fractions containing LF were pooled and dialyzed against $T_{10}E_5$ buffer (10 mM Tris and 5 mM ETDA [pH 8.0]) overnight. The dialyzed sample was concentrated by Centricon-30 (Amicon) to a volume of 1 ml. Concentrated sample was loaded onto a Sephacryl S-200 (Pharmacia) gel filtration column (100 by 1.6
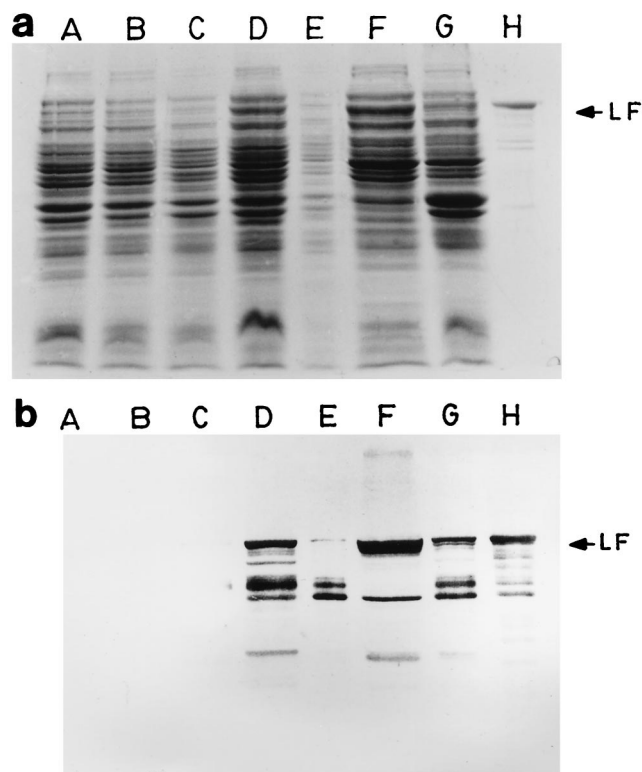

FIG. 1. Electrophoretic analysis of *E. coli*-expressed LF. (a) Proteins separated by SDS-10% PAGE and stained with Coomassie blue; (b) Western blot of the *E. coli* proteins containing LF, developed with a rabbit polyclonal LF antibody. Lanes: A, *E. coli* SG13009 cells without the vector; B, cells containing the vector pQE30 without the LF gene; C, cells containing the construct pPG-LF1 (uninduced); D, cells expressing LF; E, periplasmic proteins of cells expressing LF; F, cytosolic proteins of cells expressing LF; G, inclusion bodies of cells expressing LF; H, LF purified from *B. anthracis*.

cm) previously equilibrated with $T_{10}E_5$ buffer. Void volume was allowed to pass through, and 1-ml fractions were then collected. The rLF eluted immediately after the void volume, while the mobility of the degraded products was retarded. This resulted in approximately 90% pure rLF with a very small amount of degraded LF. The fractions containing LF were pooled and loaded onto the Resource-Q (Pharmacia) anion-exchange column previously equilibrated with $T_{10}E_5$ buffer. Protein was eluted with a linear gradient of 0 to 1 M NaCl in $T_{10}E_5$ buffer (15 ml each). Fractions of 1 ml each were collected. The protein eluted at a gradient of 250 to 300 mM NaCl. The purified LF was dialyzed against 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.0) containing 50 mM NaCl and was frozen at −70°C in aliquots. The fold purification of LF at different column stages was determined by calculating the amount of protein required to kill 50% of J774A.1 cells (50% effective concentration [EC_{50}]) when they were incubated with PA (1 μg/ml) at 37°C (Table 1). The protein was measured by the method described by Lowry et al. (17). By using this procedure, rLF was purified to homogeneity with 3,054-fold purification compared to the cytosolic preparation.

Comparison of rLF with nLF. The biological activity of rLF was compared with that of nLF purified from *B. anthracis* by the receptor binding assay, by determining in vitro binding of LF to trypsin-nicked PA, and, finally, by the macrophage lysis assay. To examine whether LF binds to receptor-bound PA,
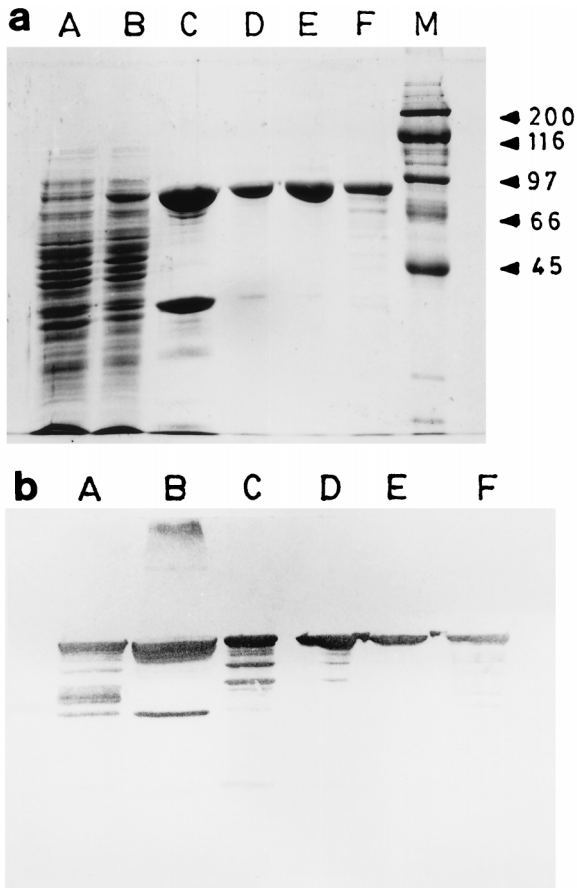
FIG. 2. Purification of *E. coli*-expressed LF. (a) Proteins analyzed on an SDS-10% PAGE gel and stained with Coomassie blue; (b) Western blot of LF proteins developed with polyclonal rabbit LF antibody. Lanes: A, *E. coli* SG13009 cells expressing the LF gene; B, cytosolic preparation of cells expressing LF; C, proteins after Ni-nitriloacetic acid affinity purification; D, protein after passing through a Sephacryl S-200 gel filtration column; E, protein after passing through a Resource-Q column on FPLC; F, LF purified from *B. anthracis*; M, molecular weight standards (10^3).

J774A.1 cells were incubated for 12 h at 4°C with PA (1 µg/ml) and radioiodinated nLF or rLF (1 µg/ml) in a 12-well plate as described earlier (3, 29). The binding of rLF to PA was 3.55 ± 0.15 ng compared to 3.7 ± 0.13 ng of nLF per mg of cell protein. Nonspecific binding in the absence of PA was 0.18 ng of the LF per mg of cell protein (Table 2).

PA cleaved by trypsin has the ability to bind to LF in solu-

TABLE 1. Purification of LF from *E. coli*

Fraction	Vol (ml)	Amt of protein (mg/ml)	Activity (EC$_{50}$)[b]	Purification (fold)[c]
Cytosol[a]	50	105.37	70.250	1
Affinity purification	10	0.61	0.042	1,672
Gel filtration	4	0.905	0.029	2,422
FPLC	2	1.5	0.023	3,054

[a] Cytosol prepared from 2 liters of culture.
[b] EC$_{50}$ is defined as the concentration of LF (in micrograms per milliliter) along with PA (1 µg/ml) required to kill 50% of the J774A.1 cells. After 3 h of incubation, viability was determined by MTT dye. The results are the means from three experiments.
[c] Purification values were determined by dividing the EC$_{50}$ for cytosol with the EC$_{50}$s for fractions obtained from different columns.

TABLE 2. Binding of LF to receptor-bound PA[a]

Protein	cpm	Amt of LF (ng)	Amt of LF/ cell protein (ng/mg)[b]
LF alone	2,160 ± 210	0.19 ± 0.02	0.18 ± 0.02[c]
PA + nLF	45,440 ± 1,260	4.05 ± 0.12	3.70 ± 0.12
PA + rLF	43,792 ± 1,620	3.91 ± 0.15	3.55 ± 0.15

[a] J774A.1 cells were incubated with 1 µg of ^{125}I-labeled LF (nLF or rLF) along with PA (1 µg/ml) for 12 h at 4°C. The cells were washed with Hanks' buffered saline solution and solubilized in 100 mM NaOH. Radioactivity was counted in a gamma counter.
[b] The protein content of the cells per well was 1.1 ± 0.05 mg as determined by the method described by Lowry et al. (17).
[c] The values are means ± standard deviations are from three individual experiments done in triplicate.

tion. Trypsin-cleaved PA molecules form oligomers, and LF molecule binds to these oligomers (20). The rLF was examined for its ability to bind to proteolytically cleaved PA (Fig. 3). Trypsin-nicked PA (1 µg/µl) was incubated either with LF (1 µg/µl) or with ^{125}I-labeled LF (100,000 cpm), and samples were analyzed on 8 to 25% polyacrylamide gradient Phast gels (Pharmacia LKB Biotechnology, Ltd.; native buffer strips). It was observed that like nLF, rLF could bind to PA and shift its mobility on nondenaturing PAGE gels.

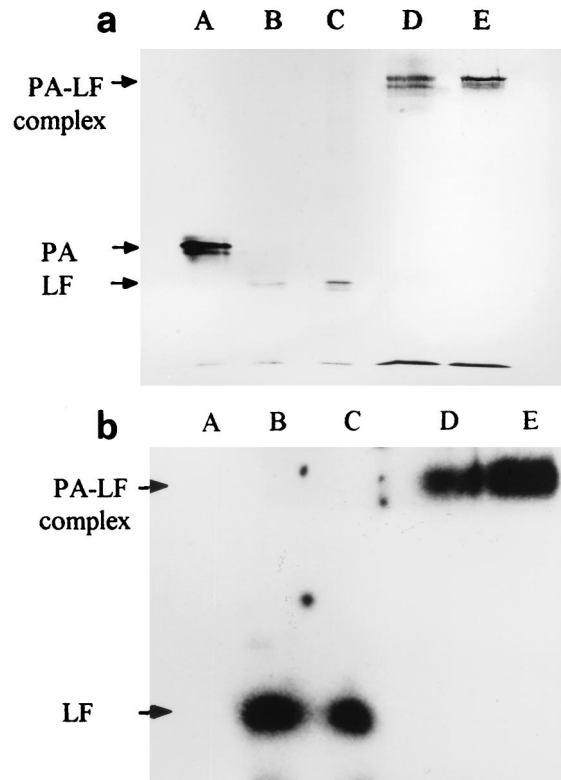rLF was also assayed for its functional activity in the J774A.1

FIG. 3. Binding of LF to PA proteins in solution. LF (1 µg) was incubated with trypsin-nicked PA (1 µg) for 15 min, and the samples were analyzed on a nondenaturing 8 to 25% Phast gradient gel. The gel was stained with Coomassie blue and dried (a), then ^{125}I-labeled LF (100,000 cpm) was incubated with trypsin-nicked PA (100 ng) and, after electrophoresis, the gel was dried and autoradiographed (b). Lanes: A, PA; B, LF from *B. anthracis*; C, LF from *E. coli*; D, PA nicked with trypsin incubated with LF from *B. anthracis*; E, PA nicked with trypsin incubated with LF from *E. coli*.

macrophage lysis assay. Cytotoxicity in response to anthrax LT was determined with 3-(4,5-dimethylthiazol-2-yl),-5-diphenyl-tetrazolium bromide (MTT) dye. Various concentrations of LF (nLF or rLF) along with PA (1 μg/ml) were added to the cells. After 3 h, viability was determined by adding MTT dye as described earlier (3, 4). rLF showed biological activity comparable to that of LF obtained from *B. anthracis* in the cytotoxicity assay. The EC_{50} of rLF was 0.023, while the EC_{50} of nLF was 0.020.

Thus, LF was purified to homogeneity by affinity chromatography, gel filtration, and fast-performance liquid chromatography (FPLC). One liter of culture yielded 1.5 mg of LF. The recombinant protein contained 12 amino acids (MRGSH HHHHHGS) added at the amino terminus of the full-length LF. These amino acids are derived from the sequence coding for the affinity tag and the sequences added by PCR manipulations. The additional amino acids had no effect on the biological activity of LF. The LF from *E. coli* behaves exactly in the same manner as the LF obtained from *B. anthracis*. Furthermore, the *E. coli* system is convenient to transform, grow, and maintain in the laboratory compared to *B. anthracis*, which requires containment facilities.

Unlike other anthrax toxin proteins, studies of LF are in their infancy. It is of interest to determine the enzymatic mechanism of action of LF. To understand the role of LF and to better characterize LF, large quantities of toxin protein are needed. The system developed in this work allows the production of large amounts of LF in pure form which can then be used for further investigations, including elucidation of three-dimensional structure and the molecular mechanism of action.

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