Physical Characteristics of rLF and rPA: Effects on Enzymatic Activity and Binding

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INTRODUCTION

Bacillus anthracis exotoxin is composed of three components; protective antigen (PA), lethal factor (LF), and edema factor (EF), which act in binary combinations. The complex of PA, the cell binding component, with LF, the metallo-endopeptidase moiety, is termed "lethal toxin" and can cause death. Recombinant forms of PA and LF are produced in an avirulent, non-capsulated, sporulation-suppressed *B. anthracis* host at List Labs.

Electrospray Ionization mass spectrometry (ESI) analysis of several lots of LF indicate that truncated forms of LF are produced, as well as the full length. An HPLC protocol was used to analyze the percent full length toxin in each lot. The percent of truncated fragments in a given sample of LF was correlated to the enzymatic activity, which is assessed by measuring the specific activity of each lot using MAPKKide™, a quenched fluorigenic peptide substrate for LF. An assay using PA_{63}-coated plates was developed to study the effect of LF length on the binding of LF to PA. In order to determine whether addition of $ZnCl_2$ would increase the enzymatic activity of LF, the kinetic parameters, K_m and V_{max}, were measured after incubation with several concentrations of $ZnCl_2$. ESI of rPA also indicated that each lot contains a truncated form as well as full length. The stability of LF and PA at room temperature was assessed using HPLC.

These studies are important for the assessment of characteristics of the proteins that may impact cytotoxicity.

MATERIALS

Recombinant lethal factor (Prod #172), protective antigen(Prod #171), MAPKKide™ (Prod #530), and MAPKKide™ Unquenched Calibration Peptide (Prod #539) are products of List Biological Laboratories.

METHODS

HPLC was performed using a strong anion exchange (SAX-10) analytical column, 4.6 x 250 mm (Dionex, Sunnyvale, CA) attached to a Varian PEEK HPLC system. Buffer A is 20 mM Tris-HCl, pH 8.0, 25 mM NaCl and Buffer B is 20 mM Tris-HCl, pH 8.0, 500 mM NaCl. A fifty minute linear gradient from 0 to 80% Buffer B, with a flow of 1ml/min was used. The column effluent was monitored at 220 and 280 nm. Details of each experiment are described in the figure legends.

Enzymatic activity, in units per mg of LF, was measured using the FRET LF substrate, MAPKKide™. One unit is defined as the amount of LF needed to catalyze the release of 1.0 µmole cleaved MAPKKide™ from intact MAPKKide ™ per min at 37°C in 20 mM HEPES, pH 8.2. MAPKKide™ is a synthetic peptide containing a single cleavage site for LF. It is a quenched fluorescent substrate peptide based on fluorescence resonance energy transfer (FRET). Initially, the N-terminally attached fluorophore, o-aminobenzoyl (Abz), is quenched by the C-terminally attached chromophore, 2,4-dinitrophenyl (Dnp). Cleavage of the substrate by LF releases the fluorophore and full fluorescence is restored. The increase in fluorescence intensity is directly proportional to the amount of cleavage that has occurred. This increase in fluorescence is measured as a function of time in this assay. The change in the relative fluorescence units (RFU) as cleavage occurs is converted to nmoles of cleaved substrate from a standard curve generated using MAPKKide™ Unquenched Calibration Peptide. This calibration peptide is the cleaved MAPPKide™ substrate containing only the N-terminally attached fluorophore, so that no quenching occurs.

Continuous fluorimetric assays were performed on a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA). The activity of each lot of LF was measured in quadruplicate using 5 nM protein. The plate was incubated at 37°C for 15 min prior to the addition of substrate. The reaction was initiated by the addition of 5 µM MAPKKide™. The time-dependent increase in fluorescence intensity was monitored at 37°C, every 45 sec, for 30 min. The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively.

LF-PA_{63} binding assay was performed using PA_{63}-coated black ELISA plates. MAPKKide™ was used to determine the amount of LF bound to the PA_{63}. Briefly, the microtiter plates are coated with 2 µg/well of PA_{63}, overnight, blocked with 2% BSA, washed, and incubated for 2 hrs at 37°C, with 10 serial dilutions of each lot of LF starting with 5 µg protein. After washing, the amount of LF bound to the PA_{63} is determined using 10 µM MAPKKide™ and the continuous fluorimetric assay described above.

LF enzymatic activity as a function of $ZnCl_2$ concentration was measured by incubating 250 nM LF in 10 mM Tris, pH 7.5, containing 100 µM $CaCl_2$ for 1 hour with varying concentrations of $ZnCl_2$. Excess unbound $ZnCl_2$ was removed by extensive desalting with a 30,000 MWCO Microcon filter. Final concentrations of protein recovered were determined using OD_{280}. Concentrations were adjusted so that the enzymatic activity was measured for 5 nM of each sample using MAPKKide™. Conversion of RFUs to nmoles was accomplished using MAPKKide™ Unquenched Calibration Peptide.

RESULTS

Electrospray Mass Spectrometry (ESI)

Several lots of LF were analyzed using ESI. The components observed in the deconvoluted spectra are shown in **Table I.** All lots of LF contain truncated forms of the protein. Since this method is not quantitative, the relative amounts of each component are not known. The recombinant form has a two amino acid extension at the N-terminus, a His and a Met, resulting in an adjusted molecular weight for LF of 90495.3.

Table I: ESI analysis of LF

LF lot #	MW of ESI components
2 (1722)	88084 89312 89448 90232 **90498**
3A1 (1723A)	89318 89458
3B1 (1723B)	89312 89448 90230 **90500**
7 (1727)	**90486**

N-Terminal Sequencing

N-Terminal sequence analysis of LF lot # 3A1 is shown in **Table II**. The amino acids found in Sequence 2 match up with residues 12-17 of the LF protein sequence. This truncated form is missing the first 11 amino acids from the N-terminal and has a MW of 89,428 Da which is consistent with the observance in ESI of a 89,458 Da component. Sequence 3 aligns with residues 13-17of LF and has a MW of 89,291 consistent with the 89,318 component in the ESI. These data indicate that the truncation of LF occurs at the N-terminal end of LF. Sequence 1 is a minor component that was only observed in the first two cycles.

Table II: N-terminal sequencing analysis

Position in LF	Amino Acid	Sequence 1	Sequence 2	Sequence 3	Sequence 4
1	H	H			
2	M	M			
3	A				
4	G				
5	G				
6	H				
7	G				
8	D				
9	V				
10	G				
11	M				
12	H		H		
13	V		V	V	
14	K		K	K	K
15	E		E	E	E
16	K		K	K	K
17	E		E	E	E

FIGURE 1: HPLC Analysis of LF

Representative anion exchange HPLC chromatograms obtained for 20 µg of LF lot #3B1 (blue), lot #7(red), lot #9 (green) monitored at 280 nm. As shown in **Table I** using ESI, recombinant LF is isolated and purified as a mixture of different length fragments ranging from 88 - 90.5 kDa. The full length protein, 90.5 kDa, can be separated from the remaining fragments using an anion exchange HPLC column. The HPLC peak labeled #1 has been identified by ESI as containing a single component of 90,498 Da, the expected molecular weight of the full length LF. The following set of overlapping peaks contain the truncated forms.

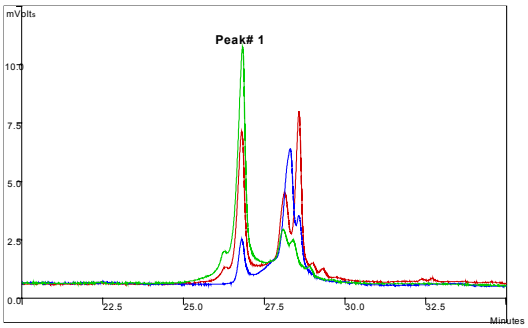
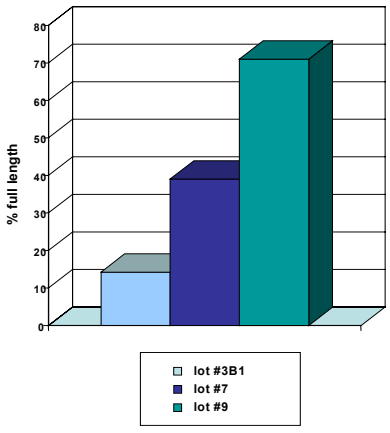


FIGURE 2: The bar graph represents the percent of the total LF intensity included in Peak #1 for each HPLC trace shown above. This represents the % of full length LF present in the sample. Lot #9 has approximately 70% full length protein. Lot #7 contains 39% full length and Lot #3B1 has the least full length LF at approximately 14%.



Measurement of LF Enzymatic Activity Using MAPKKide™

FIGURE 3: Calibration Curve generated using MAPKKide™, Unquenched Calibration Peptide for the conversion of RFU to nmoles of cleaved substrate. The calibration peptide was diluted to final concentrations of 0.2, 0.15, 0.10, 0.05, and 0.025 nmoles. The assay is run in triplicate and conditions are identical to those used for the MAPKKide™ substrate. The conversion equation is:

$$nmoles = [(RFU-A)/B]$$

where A is the x-intercept and B is the slope established from the calibration curve. The curve shown here is the average of 5 curves.

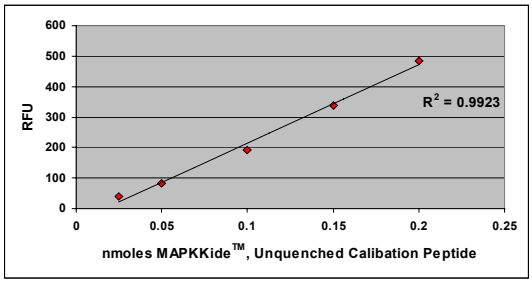
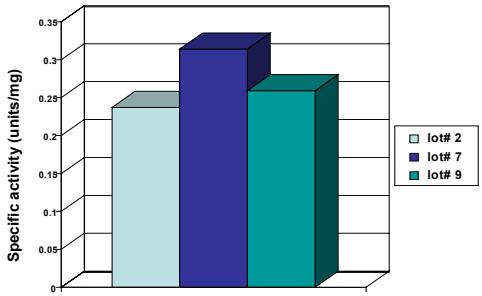
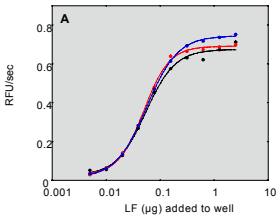


FIGURE 4: Specific activity of LF: Endopeptidase reactions with MAPKKide™ were conducted in 20 mM HEPES, pH 8.2. The specific activity in units/mg LF is shown for Lot #2, Lot #7 and Lot #9. The specific activities do not correlate with the amount of full length LF in each lot. Lot #2 has the least full length at <5%, while Lot #7 and #9 have 39 and 71%, respectively. These data suggest that the truncated forms of LF have the same enzymatic activity as the full length.

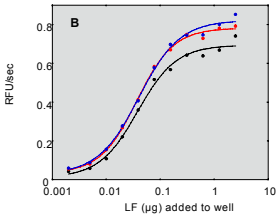


Measurement of LF Binding to PA_{63} Using MAPKKide™

FIGURE 5: Dose Response curves obtained for LF lot# 7(A), and lot #9 (B). Enzymatic activity is measured in each PA_{63}-coated well after exposure to a series of LF concentrations. The curves indicate the amount of LF bound to the PA_{63}. The black curve for both plots is the control lot #2 and the remaining curves are those obtained for two individual vials of the given lot, #7 or #9.



Comparing the curves obtained for lot #7(red and blue) with the control lot #2 (black), there is very little difference in the amount of LF bound in each well. LF lot #2 contains <5% of the full length protein, while lot #7 has 39% full length LF. These data suggest that the truncated forms of LF found in Lot #2 bind PA_{63} as well as the full length protein.

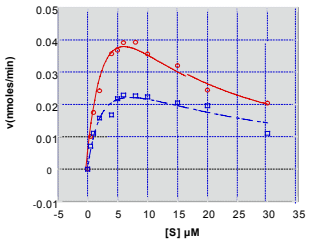


The curves obtained for Lot #9 (red and blue) are slightly displaced from the control curve (black). Lot #9 is 70% full length and it appears that there is a small increase in the amount bound to PA_{63} as compared to Lot #2.

Effect of $ZnCL_2$ concentration on K_m and V_{max} for LF cleavage of MAPKKide™

FIGURE 6: Representative kinetic curves obtained for LF pre-incubated in the presence (blue) and absence (red) of 10 µM $ZnCl_2$. The plots were obtained using KaleidaGraph 4.0. V_{max} and K_m values were calculated using the substrate inhibition equation:

$$y=(V_{max}\cdot x)/(K_m+(x\cdot(1+x/K_i)))$$

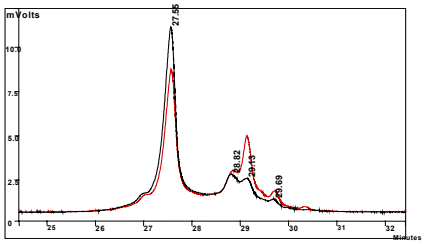


[Zn] µM	V_{max} (nmoles/min)	K_m (µM)
0	0.1	5.3
2.5	0.07	5.6
5.0	0.07	5.6
10.0	0.04	3.0

The shape of the curve clearly indicates substrate inhibition at higher MAPKKide™ concentrations, as reported previously for small LF substrates (Tonello et al, J. Biol. Chem., ***278***, 40075-40078, 2003). The data show that the V_{max} is not increased by pre-incubation with $ZnCl_2$. Previous studies in our lab also showed that addition of $ZnCl_2$ directly to the digestion buffer inhibited hydrolysis of MAPKKide™.

Stability of LF at Room Temperature

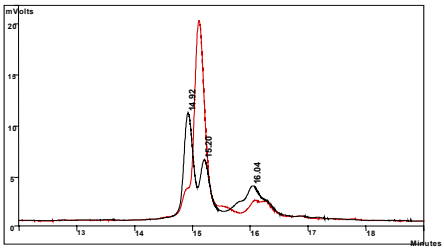
FIGURE 7: **Representative HPLC chromatograms** (280 nm) obtained for 20 µg of LF after storage for 0 (black) and 7.9 (red) days at room temperature. Conditions of the HPLC are given in the Methods Section. LF is 1.0 mg/ml in 5 mM HEPES, 50 mM NaCl, pH 7.5.



Peak at 27.55 min represents full length LF. This peak decreases and the peaks at 28.82, 29.13, and 29.69 minutes increase over the 8 days.

Stability of PA at room temperature

FIGURE 9: Representative HPLC chromatograms (280 nm) obtained for 20 µg of PA after storage for 0 (black) and 23 (red) hours at room temperature. Conditions of the HPLC are given in the Methods Section. PA is 1.0 mg/ml in 5 mM HEPES, 50 mM NaCl, pH 7.5.



The major peak (15.20 minutes) at 0 time at room temperature was shown by ESI to be the full length PA with MW of 82674 Da. The peak at 14.92 minutes accumulates as time at room temperature increases. This peak has a MW of 82603 consistent with the loss of 5 amino acids, EVKQE, from the N-terminal of PA. The effect on function of PA is unknown.

FIGURE 10: Stability of full length PA as a function of time at room temperature. There is ~ 20% degradation of the full length PA in 7.5 hrs and a significant breakdown of ~ 60% after 23 hours at room temperature.

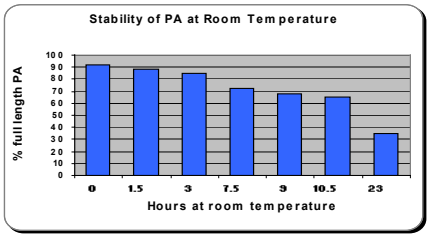
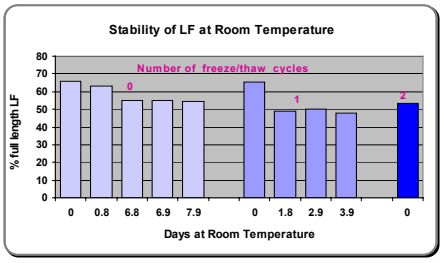


FIGURE 8: **Stability of full length LF** as a function of time at room temperature and number of freeze/thaw cycles.



LF is stable after one freeze/thaw cycle. Some degradation is observed after 2 freeze/thaw cycles. LF is stable for 0.8 days at room temperature; there is Approximately 11% degradation after 6.8 days. Degradation is slightly higher for samples maintained at room temperature, then frozen and thawed, before analysis. After one freeze/thaw, approximately 17% degradation of the full length LF is observed in 1.8 days.

CONCLUSIONS

I. Recombinant LF

A. ESI analysis of rLF indicates that the isolated protein is a mixture of fragments ranging from 88 kDa to the full length protein at 90.5 kDa. Truncation occurs at the N-terminal. HPLC analysis can be used to determine the % full length LF in a given preparation.

B. The enzymatic activity of rLF, determined using the MAPKKide™ FRET assay, is not dependent on the presence of full length protein. Similar specific activities are measured for a preparation containing <5% and one containing approximately 70% full length protein.

C. No significant change in the binding of rLF to PA63 is observed for preparations containing different amounts of full length protein.

D. The enzymatic activity of LF after pre-incubation with different concentrations of $ZnCl_2$, decreased for all concentrations of $ZnCl_2$ tested, after extensive desalting.

E. Using HPLC, it was shown that LF at 1.0 mg/ml is stable for one freeze/thaw cycle; some degradation of the full length protein is observed after two freeze/thaw cycles. LF is stable for 0.8 days at room temperature and looses approximately 11% of the full length in 6.8 days.

II. Recombinant PA

A. Using HPLC it was shown that rPA breaks down after storage at room temperature. A five amino acid fragment is lost from the N-terminal. Approximately 20% of the full length PA is degraded in 7.5 hours.