Fluorescence Substrate for Specific and Quantitative Detection of Anthrax Lethal Factor in Plasma

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ABSTRACT:

PURPOSE OF STUDY: The goal of this study was to develop a fast, sensitive, specific and accurate detection method to determine active infection by *Bacillus anthracis* in plasma. This presentation focuses on the evaluation of a fluorescently labeled peptide substrate named **MAPKKide® Plus*** for both specific cleavage by anthrax lethal factor (LF), one of the anthrax virulence factors present in blood early in infection, and resistance to cleavage by nonspecific proteases found in plasma.

METHODS USED: Two methods for the detection of LF in plasma using this fluorogenic substrate are described: one HPLC-based and one a microplate assay.

For the HPLC-based method, the LF is enriched by capture from plasma using an LF antibody-coated microtiter plate, and the captured LF is then exposed to the fluorescent substrate. The amount of cleaved peptide substrate is determined by HPLC with fluorescence detection.

Alternately the substrate may be added directly to diluted plasma in a microplate and cleavage monitored by an increase in fluorescence as a function of time using a fluorescent microplate reader.

SUMMARY OF RESULTS: Using the HPLC-based method, concentration of the LF using the antibody-coated plate allows for the detection of less than 5 pg LF/ml of neat plasma after 2 hours of incubation.

The limit of detection using the simpler microplate method is 1 ng LF/ml of plasma after 5 hours of digestion.

CONCLUSIONS: Significantly, this newly designed substrate is highly sensitive to LF and resistant to cleavage by plasma proteases making it ideal for detection of early infections with *Bacillus anthracis*.

*Patent Pending

INTRODUCTION:

Anthrax is caused by the gram-positive spore-producing bacterium *Bacillus anthracis*. The principal virulence factors are a γ-linked poly-D-glutamic acid (PGA) capsule and a three component exotoxin composed of protective antigen (PA), lethal factor (LF) and edema factor (EF). These proteins act in binary combinations. The complex of PA, the cell binding component, with the LF enzyme, is termed lethal toxin and can cause death. PA and the enzymatic EF together cause skin edema. Secreted PA is cleaved by membrane peptidases. This allows the 63 kDa carboxy terminal fragment to oligomerize to a heptamer or higher. Cleavage of PA is an essential step in exposing the binding sites for EF and LF. The complex enters the cell through endocytosis. PA mediates the transfer of LF and EF to the cytoplasm where these enzymes recognize and alter their targets.

The most lethal manifestation of *Bacillus anthracis* infection is by inhalation. Due to the intentional release of anthrax spores in the bioterrorism attacks of 2001, *B. anthracis* was placed at the top of the CDC list of select agents. Once symptoms are severe and diagnosis is possible, the levels of toxins are dangerously high. A quick, reliable test is needed to detect exposure early in the infection process.

There are a number of markers for inhalation anthrax infection including PA, LF and PGA, however, it has been shown that LF is present earliest in the infection (Boyer AE, et al, Infect Immun. **77**:3432-3441). The method described here takes advantage of the fact that anthrax LF is a zinc endoprotease which cleaves the N-terminus of mitogen-activated protein kinase kinases (MAPKK). Substantial signal amplification can be expected as a result of catalytic turnover of the substrate. Generally, peptide substrates based on the native sequences can be cleaved nonspecifically by other proteases in complex matrices such as plasma and serum. A series of fluorescently labeled peptide substrates based on the native substrate sequence were evaluated for specific cleavage by LF. Unnatural amino acids were substituted in the sequences to eliminate nonspecific cleavage.

This presentation focuses on one sequence subsequently named **MAPKKide® Plus**. The data presented demonstrate that **MAPKKide® Plus** is specific for LF and resistant to cleavage by nonspecific proteases found in plasma. Significantly, this newly designed substrate is highly sensitive to LF and may be used to detect early infections. Two methods for the detection of LF in plasma using this fluorogenic substrate are described: one HPLC-based and one a microplate assay.

MATERIALS AND METHODS:

Anthrax lethal factor (Product #169), and the chicken IgY polyclonal anti-LF antibody (Product # 769A) are products of List Biological Laboratories, Inc. The C8 Starwell Maxi Nunc-Immuno Module Plates (cat# 441653) used for LF antibody coating and the dimethyl sulfoxide (DMSO) (cat # TS-20684) were purchased from ThermoScientific. The 96-well, black, flat bottom, non binding plates used for the fluorescent plate assay were from Corning (cat # 3991). Bovine plasma (cat # 7310806) was purchased from Lampire Biological Laboratories.

Sample Preparation: Stock solutions of the fluorogenic substrate, **MAPKKide® Plus**, were 1.25 mM in DMSO based on the peptide content determined by elemental analysis. The substrate was diluted in assay buffer: 20 mM HEPES, pH 8.0 containing 0.1% Tween-20. For the microplate assays, the LF was dissolved in neat bovine plasma and diluted 1:10 in assay buffer. For the HPLC assays, the LF was added to neat bovine plasma and not diluted.

LF Activity Assays:

Microplate reader: Assays were performed on a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices). The cleavage reaction was initiated by addition of the substrate, **MAPKKide® Plus**. The concentration was optimized to minimize background fluorescence while maintaining measureable cleavage. For all experiments the time-dependent increase in fluorescence was monitored at 37°C hourly for 5 or 6 hours followed by an additional 18 to 18.5 hr overnight incubation at ambient temperature. The excitation wavelength was set to 368 nm and emission to 452 nm with a cutoff filter at 435 nm.

HPLC: The C8 Starwell Maxi Nunc-Immuno Module Plates were coated with 150 µl of a 10 µg/ml solution of a chicken affinity purified polyclonal IgY antibody to anthrax lethal factor (List Prod # 769A). Plates were incubated with the IgY overnight at 2-8°C and washed three times with 0.1M Glycine-HCl, pH 2.5. This wash was included to liberate residual LF retained after the affinity purification of the antibody and was necessary to minimize the background. After 6 washes with PBS containing 0.05% TWEEN-20 (PBST), the anti-LF coated wells were exposed to 300 µl of a series of LF concentrations in neat plasma. The plates were incubated at 22°C for 2 hours. Plates were then washed 6 times with PBST and 250 µl of 1.25 µM **MAPKKide® Plus** was added. The reaction was allowed to proceed for 2, 3.5, and 5 hours at 37°C and overnight at ambient temperature. At each time point 200 µl of the reaction mixture was removed from replicate wells and placed in HPLC sample vials.

HPLC was performed using a Zorbax Eclipse Plus C18 reverse phase column, 4.6 x 150 mm (Agilent) and a guard column containing the same resin in a Varian ProStar HPLC system (Agilent). Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. The 16 minute HPLC method was as follows: 25% B for 0.75 minutes; 25 to 45% B in 4.75 minutes; 45 to 100% B in 0.75 minutes; 100% B for 3.75 minutes; 100 to 25%B in 0.67 minutes and 5.34 minute equilibration with 25% B. The column effluent was monitored using an Hitachi fluorescence detector with excitation set to 350 nm and emission at 450 nm to detect the free coumarin fluorophore cleaved from **MAPKKide® Plus**. The injection volume was 20 µl. The 7-amido-4-methylcoumarin (AMC) peak retention time was 4.8 minutes.

RESULTS:

A. MAPKKide® Plus Specificity

The change in fluorescence for 2.5 µM **MAPKKide® Plus** in the presence of 1:10 diluted plasma without LF is compared to a peptide with the same sequence but lacking the unnatural amino acids (Control Peptide). The results confirm that **MAPKKide® Plus** is specific for LF in this system and that the peptide lacking the unnatural amino acids is non-specifically cleaved by plasma proteases (**Table 1, Figure 1**).

Table 1: Fluorescence observed for substrates in 1:10 diluted plasma without LF.

Time (hrs)	Temperature (C)	RFU	
		MAPKKide® Plus	Control Peptide
0	37	108	102
1	37	123	353
2	37	134	860
3	37	142	1420
4	37	144	1854
5	37	151	2251
6	37	147	2571
21	ambient	163	4074
39	ambient	169	3920

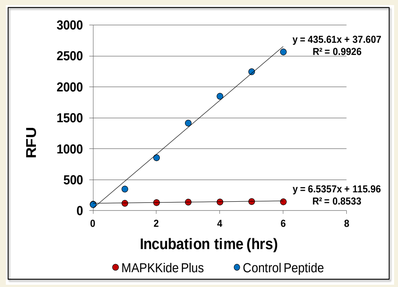

Figure 1: Fluorescence (RFU) as a function of time for **MAPKKide® Plus** (**red**) and Control Peptide (**blue**) in 1:10 plasma at 37 °C in the absence of LF.

B. HPLC Method Using Antibody Capture to Concentrate the LF.

In order to optimize the detection of low levels of LF in plasma, the LF was enriched using an affinity purified polyclonal antibody coated on a 96-well microtiter plate. In addition to enrichment of the LF, the method allowed capture of small amounts of LF from plasma without dilution. It was found that since the antibody had been affinity purified using LF there were low levels of residual LF remaining with the antibody. Several washes with 0.1M Glycine-HCl, pH 2.5 were included to liberate residual LF retained on the antibody coated plate. This wash minimized the background observed in plasma samples without LF. After addition of the **MAPKKide® Plus**, samples were monitored using HPLC with fluorescence detection after 2, 3.5 and 5 hrs of incubation at 37°C. Representative chromatograms from the digestion of **MAPKKide® Plus** by LF in neat plasma after 2 hours at 37°C are shown in **Figure 2.** The average peak areas are given in **Table 2.**

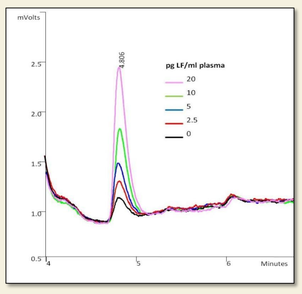

Figure 2: Chromatograms from the digestion of **MAPKKide® Plus** by 0 (black), 2.5 (red), 5 (blue), 10 (green), and 20 (pink) pg of LF/ml of neat plasma after 2 hours at 37°C.

Table 2: Detection of LF in neat plasma

LF (pg/ml plasma)	Average Peak Area*			
	2 hour digest	3.5 hour digest	5 hour digest	Overnight digest (RT)
0	2380	3840	5033	7835
2.5	3696	6294	8788	14515
5	5459	8976	12218	21377
10	8875	14448	20487	34083
20	14311	25587	34502	62133

*The average peak areas for samples containing no LF (n=6) and for samples containing 2.5, 5, 10 and 20 pg LF/ml plasma (n=2).

A plot of the peak areas as a function of LF concentration in the plasma is shown in **Figure 3**. Analysis for each response curve is given in **Table 3**. The response at each time point is linear.

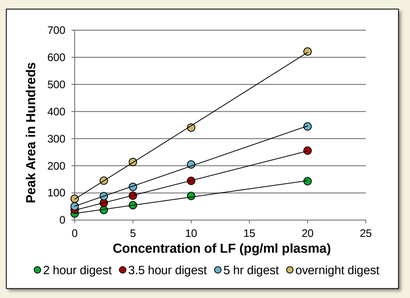

Figure 3: Plot of peak areas versus concentration of LF (pg/ml plasma) for 2 hour (green), 3.5 hour (red), 5 hour (blue) and overnight (beige) digestion of MAPKKide® Plus.

Table 3: Slope, intercept, and correlation coefficients for each incubation time.

Measurement	Incubation Time (hr)			
	2	3.5	5	Overnight (RT)
Slope (Peak Area/pg LF)	604	1093	1481	2708
Intercept (Peak area)	2411	3632	5098	7676
R^2	0.9971	0.9997	0.9991	0.9997
Std deviation of the blanks	785	817	680	469
3x standard deviation + the average	4734	6290	7074	9242
LOD (pg/ml plasma)	3.84	2.43	1.33	0.58

The limit of detection for each incubation time was estimated from the normal distribution (3 standard deviations) of blank plasma samples (0 pg LF; n=6), calculated as pg LF/ml plasma from the standard curve generated at each incubation time. This data is shown in **Table 3**. The results indicate that the limit of detection at all time points is less than 5 pg LF/ml of neat plasma.

Kinetic analysis of the LF activity by HPLC. The amount of LF in an unknown sample can be determined from any single time point as shown above. However, such results might be subject to false positives due to uncertain background fluorescence. Alternately, the results can be obtained by monitoring the reaction rate, i.e. the increase in peak area as a function of time (**Figure 4**). Each concentration of LF yields a unique slope (rate) which is proportional to the concentration (**Figure 5**). The limit of detection by this method is also around 5 pg LF/ml neat plasma.

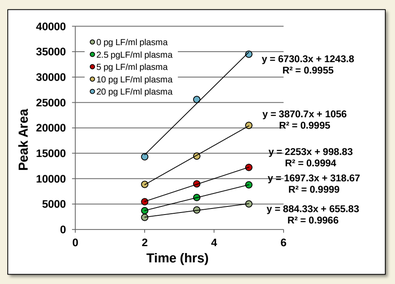

Figure 4: Plot of peak area versus incubation time for 0 (gray), 2.5 (green), 5 (red), 10 (beige), and 20 (aqua) pg LF/ml plasma.

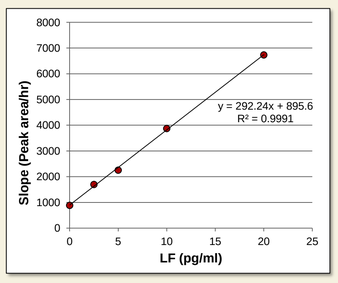

Figure 5: Plot of the slopes as a function of the concentration of LF (pg/ml plasma).

Identification of the source of background fluorescence. As seen in **Table 2** above, the average peak area observed for the blank samples that contain no LF is small but increases with time of incubation. There are three possible sources of this fluorescence background which include contributions from 1) free AMC remaining in the substrate prep after synthesis, 2) residual LF retained with the antibody after the low pH washes, 3) non specific cleavage of the **MAPKKide® Plus** by plasma proteases bound to the capture antibody. In order to estimate the relative amounts of fluorescence due to 1, 2, or 3, a series of blanks without LF were evaluated. GM6001 which is a metalloprotease inhibitor and is known to inhibit LF was used in these studies. Samples included assay buffer (AB), AB + GM6001, plasma, and plasma + GM6001 incubated at 22°C for 2 hrs with and without the LF antibody coat. **MAPKKide® Plus** was then added to each well. Any RFUs observed on non coated plates would be due to AMC or plasma proteases bound to the plate. The source of the RFUs is indicated for each sample below the column for that sample in **Table 4**.

Table 4: Identification of background fluorescence

Hours	Non-coated plates (RFU)				LF antibody-coated plates (RFU)			
	AB	AB + GM6001	Plasma	Plasma + GM6001	AB	AB + GM6001	Plasma	Plasma + GM6001
2	2164	1628	2173	2748	2393	2170	4037	2338
3.5	2590	2308	4255	2646	3973	2610	3035	3281
5	2095	2085	3755	2664	4248	2468	4921	2494
Source of RFUs	AMC	AMC	AMC + non-specific plasma proteases	AMC	AMC + residual LF	AMC	AMC + non-specific plasma proteases + residual LF	AMC

CONCLUSION:

This report describes a fast, sensitive, specific and accurate HPLC method to detect active infection by *Bacillus anthracis* in plasma at very early stages of intoxication.