MAPKKide® Plus: A Specific Substrate for Sensitive Detection of Anthrax Lethal Factor in Complex Matrices

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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 complex matrices. This presentation focuses on the evaluation of a fluorescently labeled peptide substrate, MAPKKide® Plus*.

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 bovine plasma after 2 hours of incubation. Aliquots of sheep plasma and 2% milk were also spiked with LF to determine the level of LF detectable using MAPKKide® Plus*.

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

CONCLUSIONS: Significantly, this newly designed substrate, MAPKKide® Plus*, is highly sensitive to LF and resistant to cleavage by plasma proteases making it ideal for detection of early infections with *Bacillus anthracis*. MAPKKide® Plus* can also be used to detect low levels of LF in milk.

MAPKKide® Plus* allows for both specific cleavage by anthrax lethal factor (LF), one of the anthrax virulence factors present in blood early in infection and, most importantly, resistance to cleavage by nonspecific proteases found in complex matrices including plasma.

*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.

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.

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) and sheep plasma (cat# 7319006) were purchased from Lampire Biological Laboratories. The milk (2% Lucerne) came from the local market.

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, sheep plasma or 2% milk without dilution.

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 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

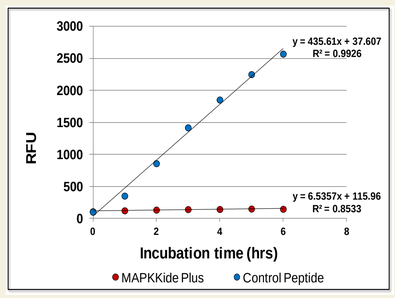

Figure 1: Fluorescence (RFU) as a function of time for **MAPKKide® Plus** (**red**) and Control Peptide (**blue**) in 1:10 plasma at 37 °C in the absence of LF.

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 or milk 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 bovine plasma after 2 hours at 37°C are shown in **Figure 2.** A plot of the peak areas obtained for the cleavage product as a function of LF concentration in bovine plasma is shown in **Figure 3**.

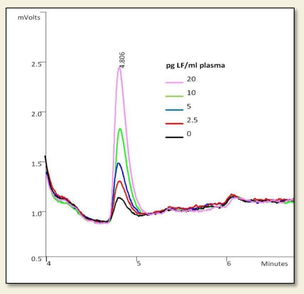

Figure 2: Chromatograms from the digestion of **MAPKKide® Plus** by 0 (black), 2.5 (red), 5 (blue), 10 (green), and 20 (pink) pg of LF/ml of neat bovine plasma after 2 hours at 37°C.

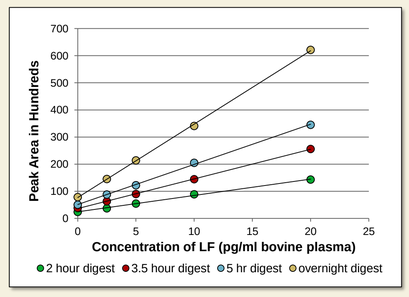

Figure 3: Plot of peak areas versus concentration of LF (pg/ml bovine plasma) for 2 hour (green), 3.5 hour (red), 5 hour (blue) and overnight (beige) digestion of MAPKKide® Plus.

The average peak areas for all three matrices evaluated are given in **Table 2.** A plot of the peak areas as a function of LF concentration in bovine or ovine plasma and 2% milk is shown in **Figure 4**.

Table 2: Detection of LF in neat bovine plasma, ovine plasma and milk.

LF (pg/ml plasma or milk)	Average peak area											
	2 hour digest			3.5 hour digest			5 hour digest			overnight digest (RT)		
	plasma		2% milk	plasma		2% milk	plasma		2% milk	plasma		2% milk
	bovine	ovine		bovine	ovine		bovine	ovine		bovine	ovine	
0	2380	3579	2781	3840	5306	3636	5033	7071	5501	7835	10678	8373
2.5	3696	5415	4441	6294	10455	6764	8788	11607	9001	14515	16268	15278
5	5459	6871	6212	8976	12210	9234	12218	14079	13241	21377	23760	23467
10	8875	9964	9694	14448	15536	15024	20487	22008	22257	34083	36140	38173
20	14311	14804	16150	25587	26007	27071	34502	36095	40754	62133	61944	66692

*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).

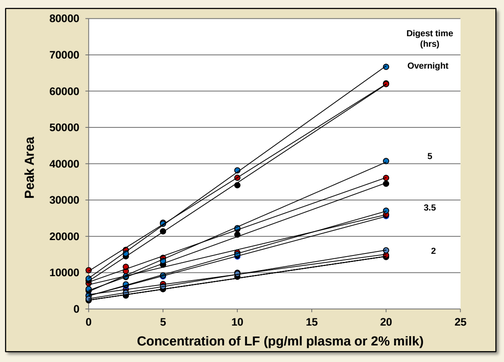

Figure 4: Plot of peak areas versus concentration of LF (pg/ml bovine plasma, black solid circle, ovine plasma, red solid circle, and 2% milk, blue solid circle) for 2 hour, 3.5 hour, 5 hour and overnight digestion of MAPKKide® Plus.

Analysis for each response curve (**see Figure 4**) is given in **Table 3**. The response at each time point as a function of concentration of LF is linear.

Table 3: Slope, intercept, and correlation coefficients obtained for **bovine plasma, ovine plasma, and milk** for each incubation time.

Measurement	Incubation Time											
	2 hour			3.5 hour			5 hour			Overnight (RT)		
	Plasma		2% milk	Plasma		2% milk	Plasma		2% milk	Plasma		2% milk
	bovine	ovine		bovine	ovine		bovine	ovine		bovine	ovine	
Slope (Peak Area/pg LF)	604	555	670	1093	965	1164	1481	1440	1782	2708	2578	2925
Intercept (Peak area)	2411	3579	2781	3632	5306	3636	5098	7071	5501	7676	10678	8373
R^2	0.997	0.994	0.999	0.999	0.980	0.999	0.999	0.999	0.999	0.999	0.999	0.999
Std deviation of the blanks	785	337	979	817	576	175	680	318	861	469	743	738
3x standard deviation + the average	4734	4589	5717	6290	7034	4161	7074	8024	8083	9242	12907	10587
LOD (pg/ml plasma)	3.8	1.1	4.3	2.4	0.4	0.5	1.3	0.5	1.9	0.58	1.0	0.7

The limit of detection for each incubation time for ovine and bovine plasma and 2% milk 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.

C. 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 5**). Each concentration of LF yields a unique slope (rate) which is proportional to the concentration (**Figure 6**). The limit of detection by this method is also around 5 pg LF/ml neat bovine plasma.

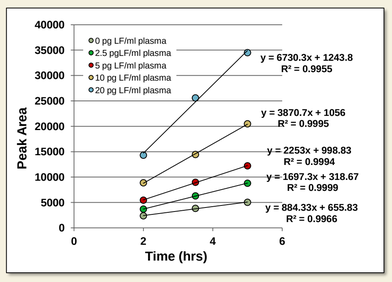

Figure 5: Plot of peak area versus incubation time for 0 (gray), 2.5 (green), 5 (red), 10 (beige), and 20 (aqua) pg LF/ml bovine plasma.

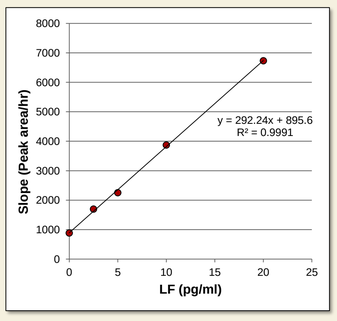

Figure 6: Plot of the slopes as a function of the concentration of LF (pg/ml bovine plasma).

CONCLUSION:

This report describes a fast, sensitive, specific and accurate HPLC method to detect active infection by *Bacillus anthracis* in both bovine and ovine plasma at very early stages of intoxication. The response at each time point (2, 3.5, 5 hours and overnight) as a function of concentration of LF is linear. The limit of detection at all time points is less than 5 pg LF/ml of neat plasma. Comparable levels of LF are detectable in 2% milk.