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A Bacteriophage-Based Platform for Rapid Trace Detection of Proteases

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Abstract: Sensitive, inexpensive, and rapid protease activity assays are of great merit for clinical diagnostics. Detection of protease-based toxins produced by *Clostridium botulinum* and *Bacillus anthracis* represents a particularly challenging task, as exceptional sensitivity is a prerequisite because of the extreme potency of the toxins. Here we present an inexpensive and sensitive assay platform for activity-based protease quantification utilizing filamentous bacteriophage as an exponentially amplifiable reporter and its application to the detection of these bacterial toxins. The assay is based on specific cleavage of bacteriophage from a solid support and its subsequent quantification by means of infectivity or quantitative PCR. Detection of botulinum neurotoxin (BoNT) serotypes A and B and anthrax lethal factor in the picomolar range was demonstrated with a limit of detection of 2 pM for BoNT/A under optimized conditions.

The action of proteases has been implicated in a large number of crucial physiological and pathological processes, and consequently, sensitive, rapid, and inexpensive protease assays are of great value for both clinical diagnostics and drug development. Timely and sensitive detection is particularly important in cases of protease-based bacterial toxins for which fast intervention is needed to prevent or ease potential intoxication, such as with the toxins produced by *Clostridium botulinum* and *Bacillus anthracis*. Botulism and anthrax are acute lethal diseases caused by these toxins, with both being listed among the six highest-risk bioterrorism threat agents by the U.S. Centers for Disease Control and Prevention (CDC). At the molecular level, a zinc-dependent protease component of either toxin cleaves key host proteins, leading to the associated morbidity and mortality. *C. botulinum* produces botulinum neurotoxins (BoNTs) that cleave proteins critical for the release of acetylcholine from neuronal cells, inhibiting neuromuscular signal transduction.[1] Similarly, the zinc protease of *B. anthracis* toxin, termed lethal factor (LF), cleaves mitogen-activated protein kinase kinases (MAPKKs), effectively disturbing macrophage signaling.[2]

Detection of these toxins represents a significant analytical challenge.[3] Because of their extreme potency [the median lethal dose of BoNT serotype A (BoNT/A) is ~1 ng/kg], extreme sensitivity is a prerequisite for any viable diagnostic. Along with the sensitivity requirements, rapid detection and the ability to discriminate between active and inactive forms of the toxin is crucial in most scenarios, including a potential response to the abuse of these toxins as bioweapons.[3] Currently, BoNT detection is accomplished via a widely used mouse bioassay that is very sensitive (up to 20 pg/mL or 0.13 pM) but exceedingly slow.[4] Alternatively, ELISA diagnostics are significantly faster and can have sensitivity approaching that of the mouse bioassay but are unable to discriminate between active and inactive forms of the toxin. While a number of alternative activity assays have been developed, none have been adopted in clinical laboratories, presumably because of the advanced instrumentation required for the signal readout [e.g., fluorescence or MALDI-TOF mass spectrometry (MS)].[3] An ideal diagnostic would incorporate aspects of both bioassays and immunoassays, that is, it would be capable of detecting *functional* toxin and employ standard procedures and instrumentation utilized in current clinical diagnostics.

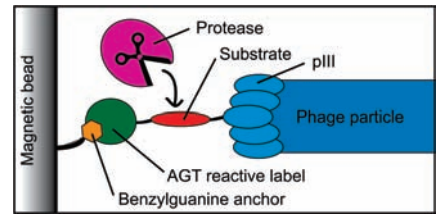

Figure 1. Phage reporter-based protease activity assay.

Here we present an inexpensive and sensitive activity-based platform for protease quantification that utilizes filamentous bacteriophage as an exponentially amplifiable reporter and its application to the detection of bioterrorism agents. While phage has been extensively exploited in the investigation and engineering of protease substrate specificity[5] as well as the protease inhibitor discovery process,[6] a phage-based quantitative protease assay has not been reported. As in other protease activity assays where the cleavage product is the analyte, inherent amplification of the output signal is achieved by the catalytic nature of substrate cleavage. However, this amplification alone is generally not sufficient for trace detection. To further increase the signal gain, a second stage of amplification was introduced utilizing postcleavage propagation of filamentous phage. The exponential nature of the phage amplification theoretically allows for the detection of as little as a single phage particle resulting from a single cleavage event.

The assay is based on release of phage from a solid support upon specific cleavage of the displayed substrate domain in the phage−solid support linker (Figure 1). The linker is displayed on the pIII phage coat protein and consists of a substrate domain and N-terminus reactive attachment domain derived from O^6-alkylguanine-DNA alkyltransferase (AGT), the latter forming a covalent bond with a benzylguanine-modified support.[7] We chose to examine BoNT/A for our initial testing because of its extreme potency and the urgent need for trace-level detection. Filamentous phage was prepared displaying a fusion of a 66 amino acid fragment of synaptosomal-associated protein of 25 kDa [SNAP-25 (141−206)][1] followed by the attachment domain. In the presence of toxin (BoNT/A light chain (LC) or holotoxin), the SNAP-25 substrate is cleaved, releasing the phage into the supernatant, and the number of phage particles released is then quantified simply by counting bacterial colonies in the presence of selection drug (Figure 2 and Figure S1 in the Supporting Information). The limits of detection (LODs) of the method, defined as three standard deviations of the blank signal,

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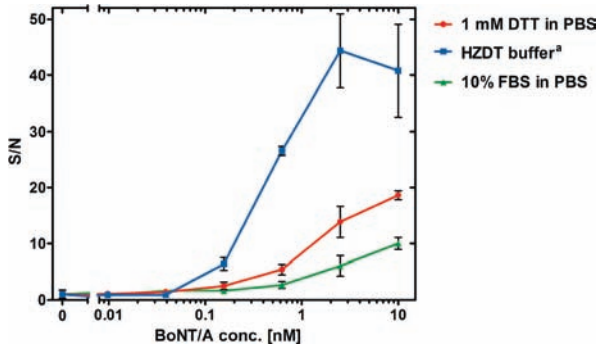

Figure 2. Relative signal intensity (signal-to-noise ratio) at various concentrations of BoNT/A. (a) HZTD buffer: 40 mM HEPES (pH 7.4), 20 μM $ZnCl_2$, 0.1% Tween 20, 1 mM DTT.

Table 1. Limits of Detection (LODs) Determined for Individual Toxins

toxin	cleavage conditions[a]	LOD (pM)
BoNT/A LC	HZTD buffer[b]	10
BoNT/A LC	10% FBS in PBS	4
BoNT/A	HZTD buffer[b]	2
BoNT/A	PBS, 1 mM DTT	120
BoNT/A	10% FBS in PBS	600
BoNT/B	HZTD buffer[b]	850
LF	0.1 M NaCl in 40 mM HEPES (pH 7.4)	690
LF	HZTD buffer[b]	2000

[a] Reactions were incubated for 3 h at 25 °C in the specified reaction buffer. [b] HZTD buffer: 40 mM HEPES (pH 7.4), 20 μM $ZnCl_2$, 0.1% Tween 20, 1 mM DTT.

were in the picomolar range, with the best sensitivity achieved being 2 pM in BoNT/A-optimized reaction buffer (Table 1). Critically, this sensitivity approaches that of the standard mouse bioassay for BoNT/A detection. It is particularly noteworthy that effective washing of untethered and/or nonspecifically bound phage from the magnetic beads prior to cleavage using high detergent concentrations (0.5% Tween 20) and elevated temperature (55 °C) was critical for enhancement of the signal-to-noise ratio (S/N).

To demonstrate the utility of this system for the detection of other toxins, synaptobrevin-phage (Syb-phage) and MAPKK-phage were constructed.[8] In a similar fashion to SNAP25-phage, Syb-phage and MAPKK-phage were attached to magnetic beads and used as sensors for BoNT/B and LF, respectively. Both proteases performed well in the assay, and the extent of cleavage was readily quantified using the colony-counting method (Figure S2). The LODs achieved with these two proteases were higher than that observed with BoNT/A, but both were within acceptable ranges for detection of the respective toxins in biological samples (Table 1). In particular, the LOD of LF (690 pM) is notable, since alternative non-MS activity-based methods for detection of LF are rare and less sensitive.[9,10]

In the case of a family of toxins such as BoNTs, the ability to quickly discriminate between serotypes is crucial, particularly as most biological therapeutics (e.g., antibodies) are tailored to the specific serotype of toxin. Importantly, this assay can readily discriminate between BoNT/A and BoNT/B, as demonstrated by the lack of cross-reactivity when the toxins were mismatched with the phage substrates (Figure S3), confirming that cleavage occurs specifically at the substrate domain.

Using colony counting as the analytical output, the assay can be completed within 16 h starting from preattached phage, which can be stored at 4 °C for several weeks (Figure S4 and Table S1). While simple colony counting is certainly advantageous from an economic

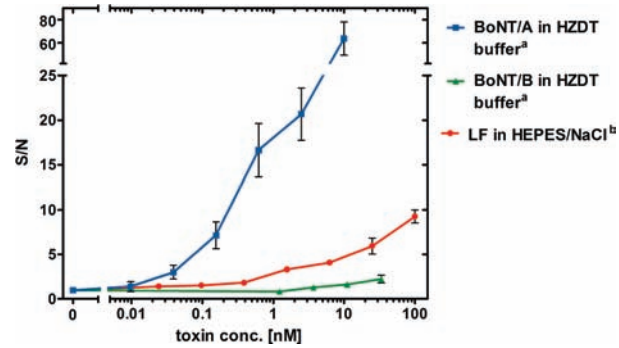

Figure 3. Relative qPCR signal intensity (signal-to-noise ratio) at various concentrations of BoNT/A, BoNT/B, and LF. (a) HZTD buffer: 40 mM HEPES (pH 7.4), 20 μM $ZnCl_2$, 0.1% Tween 20, 1 mM DTT. (b) HEPES/NaCl buffer: 40 mM HEPES (pH 7.4), 100 mM NaCl.

perspective and allows for diagnostic measurements in the absence of specialized equipment, we envisioned that use of quantitative polymerase chain reaction (qPCR) would shorten the assay time while still retaining exponential amplification of the analytical signal. Amplification of filamentous phage DNA has been used for genotyping of combinatorial library members,[11] but we are unaware of any use of qPCR for the determination of filamentous phage titer. After brief thermal denaturation of the phage particles (2 min at 95 °C), phage DNA can be precisely quantified by qPCR, showing a linear dependence of the Ct value on the logarithm of the phage input (Figure S5). With this method, output phage could be quantified with LODs and standard errors comparable to those for colony counting (Figure 3) with a significantly reduced time needed to perform the assay (~5 h).

Since a number of diagnostics currently employ qPCR as a detection modality,[12] a phage-activity-based diagnostic can be implemented clinically without the need for new or specialized instrumentation. Economically, colony counting is also very efficient, as it is estimated that the cost per reaction would be <$0.04 per assay. While the sensitivity does not entirely supplant the current mouse bioassay used for BoNT/A detection, it is critical to note that BoNT detection is a demanding example where immense sensitivity is required, particularly in the case of testing in complex matrices. If required, additional enhancement of the sensitivity and specificity can be achieved by incorporation of a pre-enrichment step, as has been performed previously.[13] Furthermore, this assay does not require the use of animals and is faster to perform, independent of a bacteria titer or qPCR readout.

The need for easily implemented, rapid diagnostic technologies for agents of bioterrorism remains large, and a bacteriophage activity diagnostic provides a powerful and robust tool for trace detection of active toxins. However, the application of this technology is not limited solely to toxins. Taking advantage of the genotype−phenotype link inherent in display technologies allows a mixture of phages to be treated with an unknown sample and the identity of the cleaved substrate, and consequently, the corresponding protease, to be deduced from the phage DNA. On this premise, we anticipate that further development will allow the application of this method to other clinical scenarios, including multiplexed profiling of panels of proteases.

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Supporting Information Available: Experimental procedures and Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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