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Rapid detection of ribosome inactivating protein toxins by mass-spectrometry-based functional assays

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This paper is dedicated to Dr. Catherine Fenselau, in recognition of her pioneering role and seminal contributions in the development of biological mass spectrometry and its applications.

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ABSTRACT

A family of structurally related protein toxins extracted from bacteria or plants have a similar mechanism of action, namely, blocking protein synthesis by ribosome inactivation. Members of this family of ribosome-inactivating proteins (RIP), classified simultaneously as chemical and biological threat agents, include ricin, abrin, and shiga toxins. We have adapted and streamlined mass spectrometry based functional assays, described by others, to develop a rapid, robust, broad-band and fieldable protocol with the primary goal of RIP toxin detection in unknown powder materials. Complementary to intact protein detection and proteomics methods, this protocol involves monitoring the depurination of selected DNA substrates by mass spectrometry. It is being implemented on the CB-TOF system for rapid detection of bio-threats by MALDI MS. Here we illustrate its performance on the example of a plant (ricin) and a bacterial (shiga) RIP toxin.

1. Introduction

A family of structurally related and potent cytosolic toxins originating from bacteria or plants have a similar mechanism of action, namely, blocking protein synthesis by ribosome inactivation [1–4]. Members of this family of ribosome-inactivating proteins (RIP) are classified simultaneously as chemical and biological threat agents. They include a number of toxins with varying toxicity (lethal dose) – ricin, abrin shiga and shiga-like toxins (verotoxin), as well as trichosanthin and luffin. Several recent events, widely publicized in the media, have underscored the need for a rapid and reliable method for RIP toxin detection in small sample amounts. Modern biological mass spectrometry is well suited for detecting and characterizing all potential bio-threat agents, including protein toxins [5–7]. Recently, new rapid methods for cleanup, concentration and separation have been developed to facilitate confident bio-threat identification by MS in complex backgrounds (soil, sea water, foodstuff, bodily fluids, etc.). For example, with suitable sample preparation protocols intact and/or digested RIP toxin molecules may be extracted and analyzed by MS (both MALDI TOF and ESI MS) [8–21]. Developments have included MS immunoassays, in which antibodies are first utilized to extract and purify the specific toxin from the mixture. Subsequently, MS analysis reveals the masses of the intact molecules attached to the antibodies. Tandem MS of the intact subunits and/or their tryptic peptides can confidently confirm the toxin identification and can also be utilized for characterization of heterogeneities. Such proteomics and antibody-purification protocols, however, may be time consuming, and more difficult to automate and use routinely in a field-laboratory setting. Furthermore, the antibodies must be sufficiently specific to capture the respective toxin without cross-reacting with contaminants. For an unknown sample, multiplexing a MS immunoassay for different RIP toxins would require multiple antibodies with the accompanying increase in cost and logistics.

Recently, a number of MS-based functional assays for detection of protein toxins (e.g., botulinum neurotoxins [22,23], anthrax lethal factor [24] and ricin [25–28]) have been described. For example, the presence of functionally active ricin in a complex sample is inferred by detection of the specific adenine cleavages induced by ricin in the respective substrate molecule (a universally conserved loop in the 28S subunit of eukaryotic rRNA). A CDC-developed protocol incorporates a three-prong approach for ricin detection: toxin immunocapture followed by monitoring the loss of an adenine group in a 12-mer synthetic DNA substrate [26]. Finally, the isolated toxin is identified by traditional bottom-up proteomics approaches. The protocol has been successfully tested for detection of ricin

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in clinical and food matrices [26]. The functional assays offer the potential for ultra-high sensitivity, since a single molecule of the target toxin can cleave multiple copies of its respective substrate, providing sufficient amplification for successful detection.

Here we describe an extension of the CDC-developed functional ricin assay [26,28] for detection of other RIP toxins. Our goal has been a broad-band functional MS detection assay for RIP toxins that, while less specific than literature methods involving antibodies, would be suitable for use in a rapid triaging setting. In real life scenarios, such an assay could and should complement other orthogonal sensors/protocols (e.g., immunoassays, [29,30]) to unequivocally confirm the presence of a protein toxin in an unknown sample before high consequence action is undertaken. Following incubation, the DNA and its cleavage products can be prepared for MALDI MS analysis via steps analogous to protocols for detection of other types of bio-threats, e.g., bacterial spores [31]. We illustrate the assay performance on the example of a plant (ricin) and bacterial (shiga) toxins. The assay is rapid (approximately 2 h total time) and allows toxin detection in picomole amounts for pure samples.

2. Experimental

2.1. CB-TOF system

We utilize the Chemical/Biological Time-of-Flight (CB-TOF) system [31] developed at the Johns Hopkins University Applied Physics Laboratory (JHU/APL). The CB-TOF is an end-to-end MS-based system for rapid triaging of white powders for *Bacillus anthracis* spores and ricin toxin. CB-TOF utilizes MALDI MS as an analytical tool to detect bio-threat characteristic signatures from unknown solid samples in less than 35 min. To characterize the CB-TOF system performance (e.g., probabilities of detection and false positive rates, and capabilities to differentiate bio-agent near-neighbors) a large number of appropriate bio-threat simulants have been examined.

CB-TOF is comprised of a JHU/APL-designed sample preparation station (SPS) for automated sample preparation, a low cost commercial TOF laser desorption mass spectrometer, and JHU/APL-written novel algorithms/software for bio-threat detection and presumptive identification [31]. A Bruker MicroFlex (Bruker Daltonics, MA) TOF instrument was used here, although the CB-TOF can be designed to accept other manufacturer's instruments as well. The JHU/APL algorithms are based on mapping of spectra of unknown samples to both experimentally acquired MS signatures and available proteomic database information. The SPS (built by Prototype Productions, Inc., VA) includes a sample cartridge with disposable plastic modules that house all necessary reagents required for MALDI MS analysis of bacterial spores and protein toxins. For intact proteins, 1 μL each of sinapinic acid matrix and sample are successively deposited on the MALDI target. The SPS accepts industry-standard 96-well MALDI targets that can be introduced into a variety of commercial MS systems for subsequent analysis. The SPS is programmed via text scripts to automatically perform a number of biochemical analysis operations and to deposit samples using a variety of sample prep protocols.

2.2. Functional assay

Commercially available protein toxin samples were used. Purified intact ricin (RCA60) and ricin A chain (RTA) were obtained from Vector Laboratories, Inc. (CA), while shiga toxin 1 (recombinant product) was obtained from List Biological Laboratories, Inc. (CA). The shiga toxin powder (10 μg) was reconstituted in 100 μL water. Sinapinic acid and 3-hydroxypicolinic acid, both used as MALDI matrixes, were obtained from Sigma Aldrich and Fluka, respectively.

In our protocol for functional detection of ricin or shiga toxins, we use two single-stranded DNA oligonucleotide substrates (5′-GCGCGAGAGCGC-3′ and 5′-CGCGCGAGAGCGCG-3′, a 12- and 14-mer, respectively). The substrates were commercially synthesized (Integrated DNA Technologies, Inc., IA) and dissolved in de-ionized water to a stock concentration of 100 μM. For ricin activity assays, the protein was dissolved in deionized water to a concentration of 7.5 μM and 1.33 μL was added to the reaction vial along with 2 μL of the DNA substrate and 16.7 μL of 10 mM ammonium citrate buffer containing 1 mM EDTA (pH 4). The sample vial containing the reactants was placed in a water bath at 45 °C for 4 h to allow reaction to proceed. After the designated reaction time, the reaction products were desalted with cation exchange beads according to standard protocols [32]. For MALDI MS analysis, 4 μL of 3HPA (431 mM in acetonitrile/water 50:50, v/v), 1 μL of reaction product mixture and 0.5 μL of 10 mM Ammonium citrate were added to a separate vial and 0.5 μL of this mixture was applied to the MALDI target for interrogation. All reactants, product, and matrices were de-salted with cation exchange beads prior to MALDI MS analysis.

The reactions of each toxin with the substrates were carried at elevated temperature in a water bath. For ricin, the reaction was optimized as a function of time and temperature and MALDI mass spectra at different time points were collected to monitor the substrate cleavage. For comparison, both positive and negative ion (linear mode) mass spectra were acquired under similar standard experimental conditions.

2.3. Safety considerations

Both ricin and shiga toxin are potent toxins, and should be handled by trained personnel only in an appropriately equipped laboratory.

3. Results and discussion

3.1. Ricin

The glycoprotein ricin is a powerful toxin that can be introduced in the body by ingestion, inhalation, or injection. It is derived from the seeds of *Ricinus communes* (castor bean) and as a byproduct of castor oil production it is easily available. Ricin is classified as a type 2 RIP. While a type 1 RIP consists of a single enzymatic protein chain, type 2 RIP are heterodimeric. Ricin is composed of two polypeptide chains of similar length – ricin toxin chain A (RTA, functionally similar to a type 1 RIP) and ricin toxin chain B (RTB). The two subunits – RTA and RTB – are held together by a single disulfide bond. The mass of the processed intact ricin molecule is around 62 kDa, while each of the protein chains is around 30 kDa. RTB binds to beta-D-galactopyranoside moieties on surface glycoproteins of mammalian cells and facilitates the RTA entry into the cell after reductively cleaving the disulfide bond. Once inside the cell, RTA functions as an N-glycosidase. It removes an adenine from the sugar phosphate backbone of an exposed hairpin in the 28S rRNA of the 60S ribosome subunit. A single ricin toxin molecule is sufficient for the complete inactivation of ribosomal function in a cell, disrupting protein synthesis and eventually cell death.

MS is well suited for the kinetic analysis of large (up to 12 kDa) RNA substrates exposed to RIP toxin subunits [33]. Several groups have reported functionally based MS assays for ricin detection and quantification in complex samples [25–28] For example, high-performance liquid chromatography and selected ion monitoring MS allowed monitoring of adenine released from a synthetic RNA

substrate by the ricin A chain (conferring the toxin's N-glycosidase activity) [34].

As part of the CB-TOF system development program [31], a MALDI-based protocol for protein toxin (ricin) detection was initially demonstrated. This protocol includes direct detection of the intact toxin in a powdered sample by MALDI MS after an-end-to-end automated sample preparation and sample analysis. Subsequently, additional non-automated detection steps have been developed that include a proteomics protocol for rapid (less than 5 min) tryptic digestion of the sample in a PCR thermocycler or by microwave heating [35]. The ricin proteolytic peptides were analyzed directly by MALDI MS (peptide mass fingerprinting) and MALDI MS/MS, and spectra were matched to the protein amino acid sequence (Supplementary Material). Applying MALDI TOF/TOF mass spectrometry and rapid trypsin digestion increases the confidence of initial positive toxin identification.

Antibody-based enrichment significantly improved the detection capability of intact ricin when mixed with several fold higher white powder backgrounds (by weight). The MS based strategies combined with antibodies provide both higher selectivity than MS alone and concentrate/purify the sample for higher sensitivity. While this combined methodology is indeed viable for MS-based ricin triage assays, its sensitivity might be jeopardized by the specificity of the antibody. Since it is generally anticipated for ricin to be found in a mixture (crude extracts from the castor bean) highly specific antibody binding in this complex background is required for unequivocal detection. Preliminary analysis using castor bean extracts revealed high non-specific binding to commercially available ricin antibodies. Proteomics analysis (Supplementary Material) has revealed the presence of ricin homologs within the crude extract, such as a castor seed coat protein, described by other groups as well.

As a complement to the CB-TOF protocols, we have tested a MALDI MS functional assay for ricin. However, in this assay we deliberately bypass the application of ricin-specific antibodies. Not relying on toxin-specific antibodies allows the functional assay to be directly applied to other RIP toxins. The assay is based on incubation of the unknown powder material into a solution containing DNA substrate strands that mimic the molecular target of ricin and other RIP. In our approach for functional detection of ricin, we expand the number of synthetic substrates to two single-stranded DNA oligonucleotides (12 and 14 bases, respectively). We have introduced two surrogate DNA targets with different masses in order to increase the assay specificity against false matches to expected cleavage product masses from unrelated molecules in the sample background. Detecting two significant peaks resulting from substrate cleavage (instead of one) improves the capability of the JHU/APL scoring algorithms. The sample-substrate solution is incubated for a specified time interval (determined by the projected sensitivity of the assay).

In order to speed up the assay, we have tested the capability of ricin to cleave the DNA substrates at elevated temperature up to 45 °C. All literature-described functional assays have been performed at 37 °C, which requires overnight incubation. Under our experimental conditions, intact ricin remains stable and functional at 45 °C (Fig. 1). Based on the observed mass spectra, the ratio of intact to cleaved material for 2 h at elevated temperature is roughly the same as the ratio for 8 h incubation at 37 °C. Furthermore, intact ricin is more active than the RTA chain alone at this elevated temperature. This might be due to the stabilizing effect of the B subunit on RTA, when the disulfide bond is intact. It is not inconsistent with observations of enhanced thermal stability of intact ricin, thus posing a challenge for its inactivation in foods [36]. Interestingly, when only one substrate (12-mer) was present, a second peak (approximately 117 *m*/*z* below the adenine cleavage peaks) is observed under typical experimental conditions (Fig. 2).

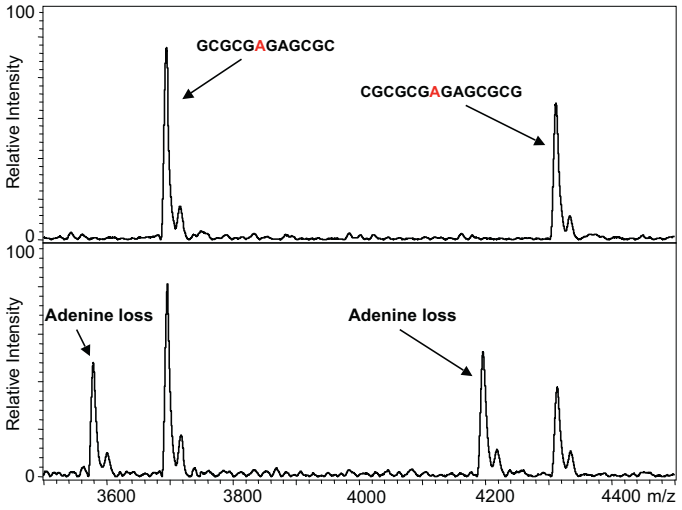

Fig. 1. Positive ion MALDI-MS spectra of reaction products of ricin incubated with two DNA substrates (12- & 14-mer): top – before reaction; bottom – after 2 h incubation time at 45 °C. The loss of adenine from the toxin substrate shifts the masses of the products, thus leading to two additional peaks consistent with this specific loss.

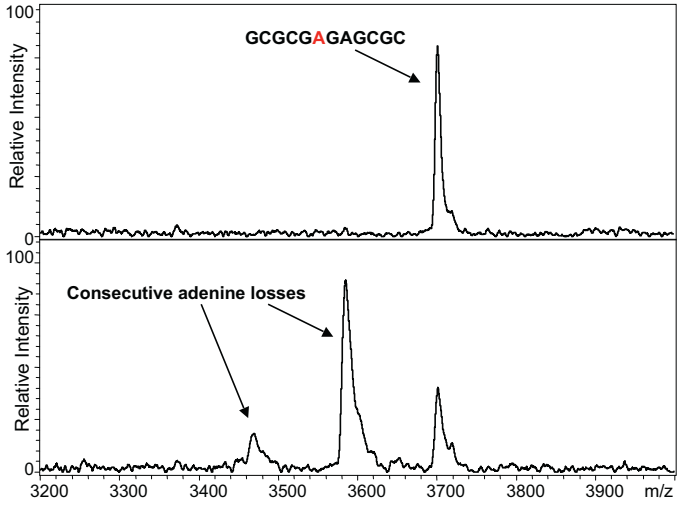

Fig. 2. Positive ion MALDI-MS spectra of reaction products of ricin incubated with only one DNA substrates (12-mer): top – before reaction; bottom – after 2 h incubation time at 45 °C. The consecutive losses of two adenines from the target substrate is consistent with the observed mass shifts.

This may be interpreted as cleavage of the second adenine in the test substrate.

3.2. Shiga toxins

Shiga and shiga-like toxins inhibit protein synthesis within a target cells by a mechanism similar to that of ricin. The holotoxin molecule is a non-covalent hexamer complex comprised of a single A-subunit (approximate MW 32 kDa) and five identical B-subunits (approximate MW for each subunit 8 kDa). The B-subunit pentamer forms a donut-shaped pore, while the A-subunit is positioned primarily on one side of the pore. The B-pentamer attaches to the cell surface and transports the A-subunit inside the cell.

Similarly to ricin, a loss of adenine is observed upon incubation of shiga toxin with the two substrates (Fig. 3). However, the ratio of intact to cleaved material does not change substantially after only a few minutes of incubation at 45 °C (we have tested the mixture

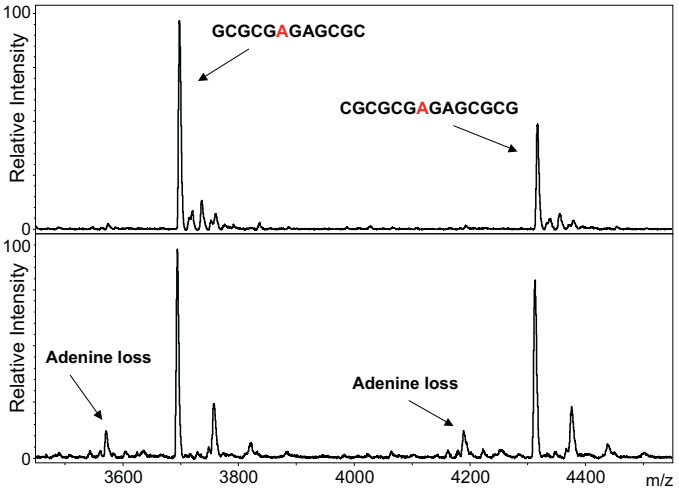

Fig. 3. Positive ion MALDI-MS spectra of reaction products of shiga toxin incubated with two DNA substrates (12- & 14-mer): top – before reaction; bottom – after 2 h incubation time at 45 °C. The two additional peaks below the respective substrates are consistent with adenine mass loss.

for up to 8 h). This suggests that the A-subunit of the shiga toxin is less thermally stable than both RTA and intact ricin.

Currently, there are more than 70 type 1 RIPs isolated from a variety of higher plants. For example, tritin – a type 1 RIP – has been identified as an abundant protein in flour [37]. To avoid misidentifications of a possible bio-threat (and the associated consequences of a false alarm), a multiplexed analytical approach that includes in addition to the functional assay, described here, direct MS detection after appropriate purification and subsequent bottom-up MS/MS verification is warranted.

4. Conclusions and future work

We have developed a rapid broad-band functional assay for detection of RIP toxins from bacterial or plant origin. The assay is easy to perform, requires a simple controlled heating device (e.g., a PCR thermocycler) and it takes about 150 min end-to-end. Detecting a signal from two DNA substrates with different masses improves the assay robustness. We plan to demonstrate the assay with other RIP toxins (abrin), and further optimize matrix-sample deposition for end-to-end automated CB-TOF analysis including the sample prep station. While the assay has been developed for MALDI MS, it can potentially be combined with other ionization techniques/mass analyzers, e.g., electrospray and DESI MS. Finally, the assay protocol will be transferred to an outside lab for additional testing and evaluation, e.g., effect of contaminants, crude extracts, sensitivity, etc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2011.08.012.

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