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Improved Sensitivity for the Qualitative and Quantitative Analysis of Active Ricin by MALDI-TOF Mass Spectrometry

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Abstract

Ricin is a highly toxic protein which causes cell death by blocking protein synthesis and is considered a potential bioterrorism agent. Rapid and sensitive detection of ricin toxin in various types of sample matrices is needed as an emergency requirement for public health and anti-bioterrorism response. An *in vitro* MALDI TOF MS-based activity assay that detects ricin mediated depurination of synthetic substrate was improved through optimization of the substrate, reaction conditions and sample preparation. In this method, the ricin is captured by an specific polycolonal antibody followed by hydrolysis reaction. The ricin activity is determined by detecting the unique cleavage product of synthetic oligomer substrates. The detection of depurinated substrate was enhanced by using a more efficient RNA substrate and optimizing buffer components, pH and reaction temperature. In addition, the factors involved in mass spectrometry analysis, such as MALDI matrix, plate and sample preparation, were also investigated to improve the ionization of the depurinated product and assay reproducibility. With optimized parameters, the limit of detection of 0.2 ng/mL of ricin spiked in buffer and milk was accomplished, representing more than two orders of magnitude enhancement in assay sensitivity. Improving assay's ruggeddness or reporducibility also made it possible to quantitatively detect active ricin with a three order of magnitude dynamic range.

INTRODUCTION

Ricin is a protein toxin produced by the seeds of the castor bean plant (*Ricinis communis*). It belongs to the family of type II ribosome-inactivating proteins, which inhibit protein synthesis by depurinating a specific adenosine (A4324 in mammals) from 28S rRNA [1–3]. The cytotoxin, ricin, is a disulfine-bond linked dichain molecule containing a catalytic A chain (RTA), which exhibits glycosidase activity, and a lectin B chain that facilitates cellular uptake. Ricin is a highly toxic substance which possesses a very low lethal dose limit with an estimated LD_{50} to be 5– 10μg/kg body weight in humans by inhalation [4]. Castor beans are common plants and the procedure of extracting ricin from castor seeds is relatively easy. This property plus the high toxicity makes ricin a potential bioterrorism agent and was classified as a select agent by the Centers for Disease Control and Prevention (CDC)[5] and a schedula A toxic chemical by the Chemical Weapons Convention.

Disclaimer: Opinions, interpretations, conclusions and recommendations in this report are those of the authors and not necessarily endorsed by the Centers for Disease Control and Prevention.

A rapid, sensitive and reliable method for the detection of ricin toxin in various types of sample matrices is necessary for the protection of human health and anti-bioterrorism purposes. Numbers of methods have been reported including assays that can only identify the presence of ricin but not its glycosidase activity, as well as assays that are able to detect biologically active ricin [6]. In non-functional assays, the ricin protein can be detected by enzyme-linked immunosorbent assay (ELISA)[7,8], polymerase chain reaction (PCR)[9], surface plasmon resonamce [10], mass spectrometry[11,12] or other techniques. To detect functionally active ricin, two types of methodology have been used, monitoring RTA-induced inhibiton or measuring the release of adenine or depurinated product from the RTA cleaved substrate while the latter one is more convenient. In the functional assays where hydrolysis product is monitored, the ricin is usually captured or enriched from environmental samples by immunoaffinity technique and then reacted with a substrate. The release of labeled or unlabeled free adenine from ribosome or synthetic oligonucleotide substrate can be detected by various detection techniques including liquid scintillation counting [13], chemiluminescenece[14], HPLC [15], or mass spectrometry[16–18].

Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-MS) is a useful tool for detecting analytes in complex samples due to its high-throughput properties and high tolerance of impurities. This technique has been utilized in our and other laboratories for the rapid and sensitive detection of ricin using *in vitro* activity assays or enzymatic digestion of the toxin [11,16,19]. We previously reported on an activity assay for ricin in which, the depurinated product from a 12-mer DNA substrate is cleaved by immunocaptured ricin and detected by MALDI-MS. The presence of active toxin in a sample can be demonstrated by the appearance of a new peak, bearing the accurate mass of the depurinated substrate in the mass spectrum of the sample, but not in that of the negative control, after the completion of the RTA cleavage reaction. In this report, we describe the improvement of this MALDI-MS based activity assay by means of optimizing the reaction conditions, MALDI matrix, and sample spotting; which led to elevated sensitivity and a potential for quantitative analysis of active ricin toxin.

EXPERIMENTAL PROCEDURES

Materials

Ricin and polyclonal goat anti-*ricinus communis* antibody (catalog number: AS-2084) were obtained from Vector Laboratories (Burlingame, CA). Streptavidin-coupled Dynabeads (M280) were purchased from Invitrogen (Lake Success, NY). EZ-link Sulfo-NHS-Biotin was obtained from ThermoFisher Scientific (Waltham, MA). The 14-mer RNA substrate (CGCGCGAGAGCGCG), 12-mer DNA substrate (GCGCGAGAGCGC) and other oligonucleotides were synthesized by Integrated DNA Technologies (IDT) (Coralville, IA). μFocus MALDI sample plate (900 μm) was purchased from Hudson Surface Technology (Old Tappan, NJ). All chemicals were obtained from Sigma–Aldrich (St. Louis, MO) except where otherwise indicated. Acetonitrile was purchased from Burdick and Jackson (Muskegon, MI). Milk (2%) was purchased from a local grocery store.

Antibody capture of ricin

The polycolonal antibody was biotinylated using EZ-link Sulfo-NHS-Biotin and then immobilized to the streptavidin-coupled Dynabeads according to manufacturers' instructions with minor modifications. The toxin spiked in 0.5 mL of phosphate-buffered saline with tween (PBST) or milk was mixed with an aliquot of 20 μL of antibody-coated beads for 1 hr with constant agitation or in an automated fashion using the KingFisher96 magnetic bead processor (ThermoFisher Scientific). Following antibody capture, the beads were then washed twice with 1 mL of PBST, twice with 0.5 mL of PBST, and then washed once in 100 μL of water.

Ricin activity reaction

The optimized reaction buffer consisted of 5 mM ammonium citrate with 1 mM ethylenediaminetetraacetic acid (EDTA) adjusted to a pH of 4.1. The substrate of 12-mer DNA, 14-mer RNA or other synthetic oligomers was reconstituted to 1 mM in deionized water. Other reaction buffer systems were used with different components and concentrations as indicated in the text. Ricin arrived as a solution of 5 mg/mL in 10 mM phosphate, 0.15 M NaCl, pH 7.8, and 0.08% sodium azide and was diluted to working concentrations in the reaction buffer. Reaction solutions consisted of the toxin bound antibody-coated magnetic beads in 18 μL of reaction buffer and 2 μL of substrate solution at the final concentration of 100μM. All samples were then incubated at 37°C (or other temperature as indicated in text) for 0.5 hr or 4 hr without agitation.

MALDI MS analysis

Aliquots from the ricin activity reaction were analyzed by a MALDI-TOF mass spectrometer. After the incubation, 2 μL of the supernatant was removed and added to 18 μL of the MALDI matrix solution. The optimal matrix solution consisted of 250 mM 3-hydroxypicolinic acid, 40 mM ammonium citrate and 5 mM ammonium tartrate in 50/50 acetonitrile/water. Other components of the matrix solution were examined, including concentrations, and they are indicted in the text. A 0.7 μL aliquot of the sample/matrix mixture was then applied to the 384-spot μFocus MALDI plate in triplicate. The spots were either air dried or dried under vacuum at −25 psi for 5 min in a 96-well plate extraction manifold (Waters, Milford, MA). Mass spectra of each spot were obtained by scanning from 1000 to 5000 m/z in MS-positive ion linear mode on model 4800 or 5800 MALDI-TOF/TOF MS Proteomics Analyzer (Applied Biosystems, Framingham, MA). The spectra were processed using the masses of the substrate and depurinated product as internal mass calibrants, to adjust the mass accuracy of the instrument in the linear mode. The ratio of the peak areas of the product and the remaining unreacted substrate were used for quantitative analysis. The instrument uses a nitrogen laser at 337 nm, and each spectrum is an average of 2400 laser shots. The data were usually an average of three experiments. CV values for the samples were typically at or below 20%.

RESULTS AND DISCUSSION

Evaluation of synthetic DNA and RNA substrates

Synthetic DNA and RNA oligonucleotides have been used as effective substrates for ricin cleavage in various *in vitro* ricin activity assays[20]. To improve the sensitivity of our MALDI-MS-based ricin activity assay and to develop the assay to be an effective, robust quantitative tool, we tested several synthetic DNA or RNA oligomers for potential depurination substrates and the results from some selected substrates are listed in Table 1. DNA12 is a 12-mer DNA fragment mimicking the loop-stem structure of 28S subunit of rRNA that is the in vivo site of ricin mediated depurination and has been designed as the effective substrate in our assay [16]. RNA14, a 14-mer ribonucleic acid, possesses a higher catalytic turnover than its DNA counterpart [15,21] and serves as a substrate in an electrospray ionization mass spectrometric method (ESI-MS) where the adenine released from the depurination of the substrate is analyzed[18]. The cleavage products from these two oligomers were evaluated and their efficiency as suitable substrates in the assay was compared. As displayed in the MALDI spectra (Fig. 1), both substrates yield peaks corresponding to their depurinated products. However, the peak area ratio of the product versus unreacted substrate from the sample with RNA14 (15.5) is much higher than that (0.4) of DNA12 (Table 1). In a MALDI-MS mass spectrum, the peak intensity of a depurinated product and the relative product/substrate ratio reflects the combinational effect of substrate depurination activity and ionization efficiency of the product. This difference in the peak ratio between the two substrates might be caused by the higher depurination activity of the RNA substrate or more ionization of the RNA product or both. On the other hand, a small peak corresponding to the mass of the cleavage product was observed in the spectrum of the control sample with DNA12 although no toxin was present (Fig. 1A) This suggests that it was formed by a nonspecific cleavage under the experimental conditions used, an known phenomenon for a small DNA oligomer, presumably caused by acid cleavage at the high incubation temperature. Although RNA oligomers are susceptible to RNases, the much higher response of the ricin cleavage reaction and a non-detectable blank peak with antibody captured ricin prompted us to adopt RNA14 as a new substrate in our MALDI-MS assay. It should be noted that the product/substrate peak ratio was used in this paper, but not the addition of an isotope labeled internal standard, for the comparison of the relative production of the ricin cleavage reaction. This is because the instrument mass resolution in the linear mode is relatively low and there is not enough mass resolution to distinquish the the analyte from an internal standard where usually a few dalton mass is added. In addition, the sequence of the depurinated product is highly similar to that of the substrate, as only small part (adenine, 118 Da) is removed from the RNA14 (4538 Da). Therefore, the ionization efficiencies of these two molecules are speculated to be similar. For this reason, we considered the area ratio of the product peak and unreacted substrate peak in the MALDI mass spectra a suitable parameter for quantitative comparison of active ricin detection.

Although it has been demonstrated that the first adenosine of the GAGA tetraloop of the 28S rRNA is the only nucleotide that is modified by ricin during the inhibition of protein translation[2], the observation of possible consecutive adenine losses from a synthetic 12-mer DNA substrates implied that the depurination might occur on another other adenosine in the

GAGA motif of a short oligomer as well [19]. If this is the case, increasing the number of adenosines by extending this specific motif could lead to cleavage on multiple sites and this might bring an increase in the sensitivity of ricin detection. To test this hypothesis, two new RNA fragments, RNA16-3GA and RNA16-4GA, were designed where the GAGA loop was replaced by a GAGAGA and a GAGAGAGA unit, respectively, whereas the stem part remained unchanged (Table 1). It was found that, however, these oligomers produced much lower cleavage fragments than that of the RNA14. The yield of the 16-mer RNA with the GAGA unit was lower than that of RNA14, but much higher than the cleavage of the RNA16-3GA and RNA16-4GA, suggesting that the sequence of the extra GA had a significant impact on ricin activity, presumably by disrupting the loop structure of those substrates. Another attempt to improve substrate stability was made by replacing regular nucleotides in the RNA14 with 2′-O-methyl-RNA ($RNA_{Me}14$) and 2′-fluoro-RNA (RNA_F14) residues, because the 2′-O-methyl RNA and 2′fluoro RNA prove to be useful RNA analogs that are resistant to general base hydrolysis and nucleases [22–25]. The incubation of these two oligomers with ricin, however, did not generate any visible cleavage peaks in the MALDI spectra and only a small amount of released adenine was detected (Table 1), revealing those analogs could not serve as suitable ricin substrates. Taken together, the RNA14 was used as the substrate for assay optimization.

Optimization of the conditions for the ricin depurination reaction

Common components in the reaction buffer of an *in vitro* ricin activity assay include citrate or acetate salts since ricin and other ribosome inactivating proteins show maximum enzymatic activity with synthetic DNA or RNA substrates at acidic conditions [20]. In an attempt to improve assay sensitivity, we examined the effect of reaction buffer on the RTA cleavage reactions. Several reaction solutions were prepared with ammonium or sodium salts of citric acid or acetic acid, and the pH of all solutions was adjusted to 4.0. Although no metal ion is demonstrated to be involved in the catalytic mechanism of ricin depurination[26], it has been reported that ethylenediaminetetraacetic acid (EDTA) seems to play a role in the *in vitro* activity assay [27]. Therefore, this reagent was added into some of the reaction solutions for evaluation. Figure 2A depicts the results produced from the depurination reaction conducted in eight buffered solutions. It was observed that the buffers consisting of ammonium citrate with or without 1 mM EDTA yielded the highest level of product while the sodium salt based buffers, with the exception of sodium acetate generated a reduced amount of products. The underlying mechanism for these variations is not clear, but this data reveals that careful selection of the reaction buffer components is necessary. Further experiments with various concentrations of the ammonium citrate salt demonstrated that 5 mM was the optimal buffer concentration (Figure 2B). Lower amounts of the salt (1mM or 2mM) in the reaction solution decreased the detection of the cleavage product, presumably due to reduced buffering capacity. On the other hand, higher salt concentrations also led to reduced cleavage, the reaction in 50mM ammonium citrate only produced half of the product intensities and the cleavage was inhibited by 90% using the 100mM reaction buffer.

Unlike the depurination of an intact ribosome substrate occurring under neutral condition, the RTA hydrolysis of the short tetraloop oligonucleotide favors an acidic environment (pH4.0)[15]. To evaluate the pH dependence of the product intensity in our MALDI-MS

platform, the effect of buffer pH was reexamined with a very narrow pH range of 3.0 to 5.0. While the optimal pH determined here (4.0 and 4.2) is consistent with the number reported previously, it was surprising to observe that the tolerance window of the pH change was extremely small (Figure 3). When the buffer pH decreased to 3.8 or increased to 4.4, only a shift of 0.2 pH units, the product-to-substrate ratio decreased by approximately 30% and 50%, respectively, representing a significant reduction in the assay sensitivity. Further changes in pH by another 0.2 units (below 3.6 or at and above 4.6) led to an almost undetectable cleavage product under the reaction conditions tested, underlining the importance of strict control of the reaction pH.

Another factor that may affect the reaction rate and yield is the reaction temperature. It has been reported that ricin is active to a 2551 bp DNA substrate from 30 to 85°C and optimal temperature is 68°C[28]. To explore whether a temperature higher than 37°C, currently used in the assay, could improve the performance of our assay, the temperature dependence of the RTA cleavage for the RNA14 substrate was examined using a range of 37°C to 75°C. Figure 4 displays that the relative intensity of the depurinated product at 4420 Da, versus that of unreacted substrate peak at 4538 Da, gradually increased from 37°C to 55°C, indicating an elevated cleavage rate with increasing temperature. This trend, however, was not observed at higher temperatures where the product-to-substrate ratio underwent a slight decline at 60°C and a dramatic drop was observed with further increase of the reaction temperature, until almost no detectable product peak at was seen at 75°C. This data indicates a reaction temperature in excess of 60°C negatively impacted the hydrolysis reaction, probably due to high-temperature induced comfomational change or degradation of either the ricin enzyme or short oligomer substrate. For this reason, 55°C was chosen as the optimal reaction temperature for future experiments.

Improvement of MALDI-MS detection of the ricin cleavage product

MALDI mass spectrometry is a routine method for the analysis of peptides and proteins but still holds challenges for the analysis of oligonucleotides like DNA and RNA oligomers[29]. Several factors that may affect the signal response and reproducibility were investigated including MALDI matrix composition, sample plate and sample spotting method in order to lower the limit of detection of our assay and to allow the reproducible quantification of biologically active ricin.

3-hydroxypicolinic acid (HPA) is the most common MALDI matrix for DNA and RNA analytes and was the matrix used in our previous MALDI-MS based ricin activity assay. A saturated HPA solution in 50% acetonitrile and water is usually used. To investigate whether the components of HPA matrix affect the detection of the depurinated product, various combinations of the concentrations of HPA and two other components, ammonium citrate and ammonium tartrate, were examined. The relative production detected from different MALDI matrix solutions is listed in Table 2. Regarding ammonium citrate, it was observed that the addition of this compound (No. 1 to No. 4) in the matrix enhanced the product-to-substrate ratios and up to a two-fold improvement was achieved when a concentration of 80 mM ammonium citrate was present. In addition, a significant improvement was also attained when the concentration of 3-HPA was reduced by half from 0.5 M (No. 3, 0.061) to 0.25

M(No. 5, 0.119), suggesting that the concentration either promotes the ionization of depurinated RNA14 or suppresses the signal of the unreacted substrate or both. Meanwhile, the amount of ammonium tartrate in the matrix solution does not seem to have any effect on the product-to-substrate ratio(No. 5 and No. 6). On the other hand, no product was detected at a lower matrix concentration of 3-HPA (0.1 M), demonstrating the requirement of a sufficent amount of matrix molecule for the detection of this specific analyte. Based on this result, the combination of 0.25 M 3-HPA, 40mM ammonium citrate and 10 mM ammonium tartrate in 50% acetonitrile was chosen as the optimized matrix solution.

The spot-to-spot variations caused by inhomogeneity in MALDI dried droplet spots limit the application of MALDI for quantitative analysis of biomolecules and this becomes more serious for oligonucleotide characterization[29]. To overcome this limitation, a number of approaches have been reported including hydrophilic anchor plates, new matrixes and novel sample preparation methods[30,31]. When the reaction solution of RTA depurination products together with the HPA matrix was applied to a hydrophilic anchor plate, improved ion signals of the analytes and high quality mass spectra were obtained compared to the samples applied to a regular stainless steel MALDI plate (data not shown). Additional improvement was accomplished by replacing the regular air-drying method with a vacuum-drying method in which the sample solution deposited on a MALDI plate was dried under vacuum using a small solid phase manifold. It has been reported that drying the spots by vacuum facilitates the formation of more homogenous spots and thus improves reproducibility [32]. Table 3 lists the results for the detection of ricin cleavage products using air-drying versus vacuum-drying of MALDI spots. While most of the air-drying triplicate spots, under two different toxin levels, produced percent coefficients of variation around 8 to 24%, all of the vacuum-dried samples generated lower than 5% coefficients of variation, demonstrating a dramatic improvement in spot-to-spot variation and reproducibility.

Improved assay sensitivity and quantitative analysis of ricin activity

With the improved efficiency for the ricin cleavage reaction and enhanced sample reproducibility of MALDI-TOF MS analysis, the next step is to examine the assay sensitivity and explore the feasibility of quantitative characterization of ricin toxin present in samples through the *in-vitro* activity assay platform. The response of cleavage products from the reactions of various concentrations of ricin spiked in PBST buffer and 2% commercial milk are displayed in figure 5. The aliquots of the reaction solutions after 0.5 hour and 4 hours incubation time were subjected to MALDI analysis so that an extended dynamic range could be achieved. A linear range of the standard curve from 2 to 500 ng/mL ricin in both buffer and milk media was observed from a 30 min reaction and a 0.1 to 10 ng/mL dynamic range for the 4 hours time point. The greater than 0.99 R^2 values suggest that this MALDI-MS based assay could be used to effectively quantify the presence of active ricin in samples within the dynamic range of three orders of magnitude. In addition, the limit of detection of 0.2 ng/mL (or 3.3 fmol/mL, Signal/noise > 3) represents more than two orders of magnitute enhancement in assay sensitivity compared with the LOD of 2 pmol/mL before improvement [16].

CONCLUSION

The MALDI-TOF MS based *in vitro* ricin activity assay was improved through optimization of substrate, reaction conditions and sample preparation. A 14-mer RNA was selected to replace the 12-mer DNA substrate to increase RTA hydrolysis efficiency. The buffer constituents, pH and temperature were optimized to promote the detection of more of the depurinated product. In addition, the factors involved in the mass spectrometry analysis, such as MALDI matrix and plate and sample preparation, were investigated to obtain improved ionization of the cleavage product and reproducibility. Using the optimized conditions, more than two orders of magnitude enhancement in assay sensitivity was achieved and the high reproducability of the data made it possible to perform quatitative analysis of ricin activity in samples. This method would prove to be a useful tool for a quick, high throughput detection and quantification of ricin toxin in samples for public health and counterterrorism purposes.

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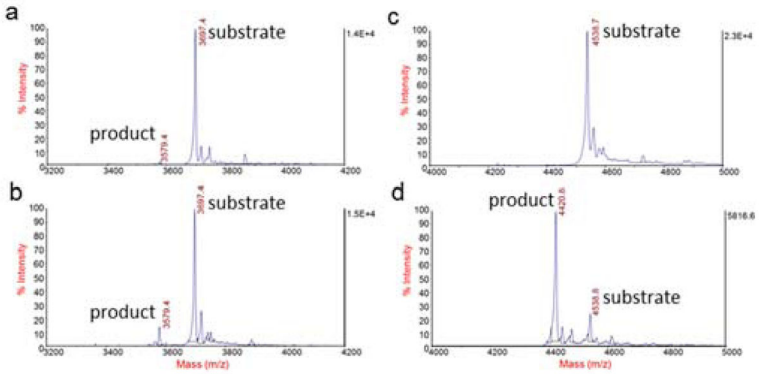


Figure 1.

Mass spectra of the cleavage reaction using DNA12 as a substrate in the absence (a) and presence (b) of ricin, and using RNA14 substrate without (c) and with (d) ricin. Reaction: 0 or 100 ng ricin, 83 uM substrate, pH 4.0, 55°C for 2 hrs. Sub and prod denote unreacted substrate and cleavage product, respectively.

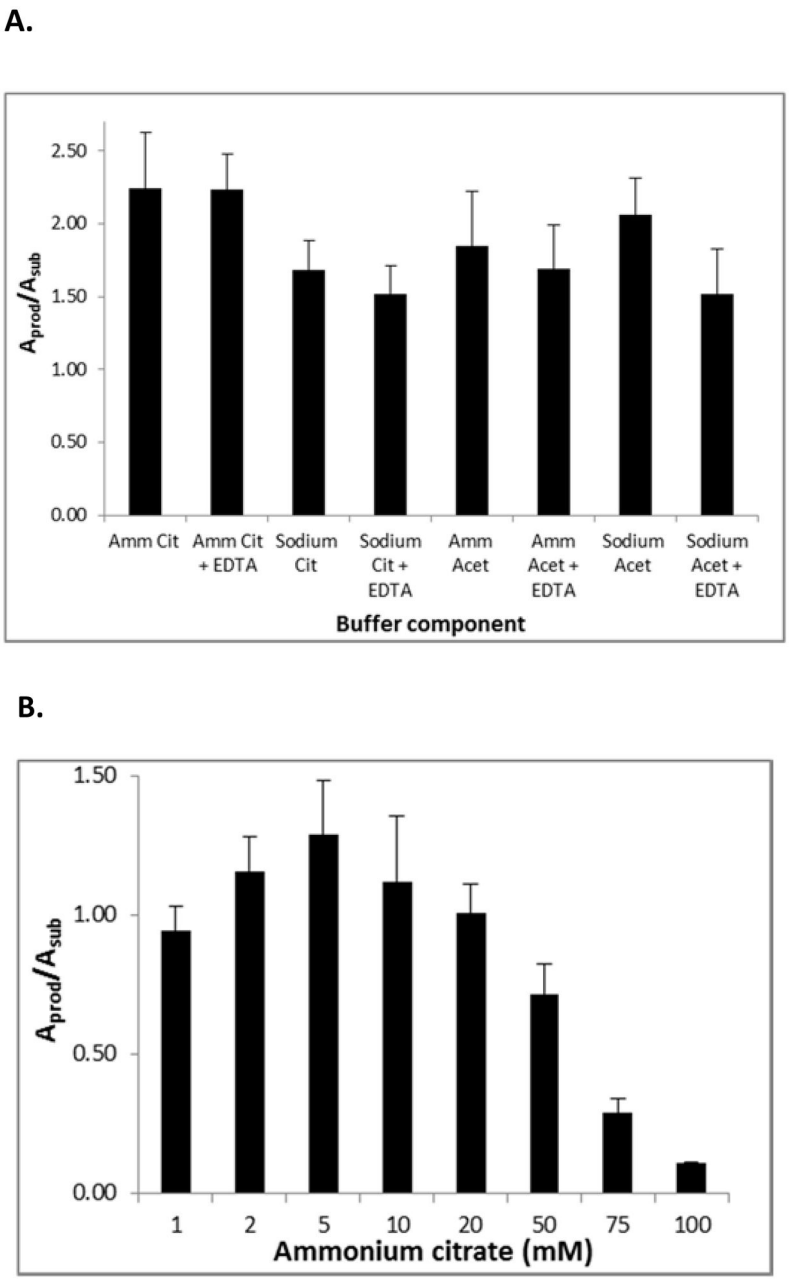


Figure 2.

(A) Relative product formation from the cleavage of the RNA substrate by ricin with various reaction buffers (10mM salt and 1mM EDTA, pH 4.0, 45°C/1hr). (B) Effect of ammonium citrate concentration on the cleavage reaction. Amm, Cit, and Acet represent ammonium, citrate, and acetate, respectively.

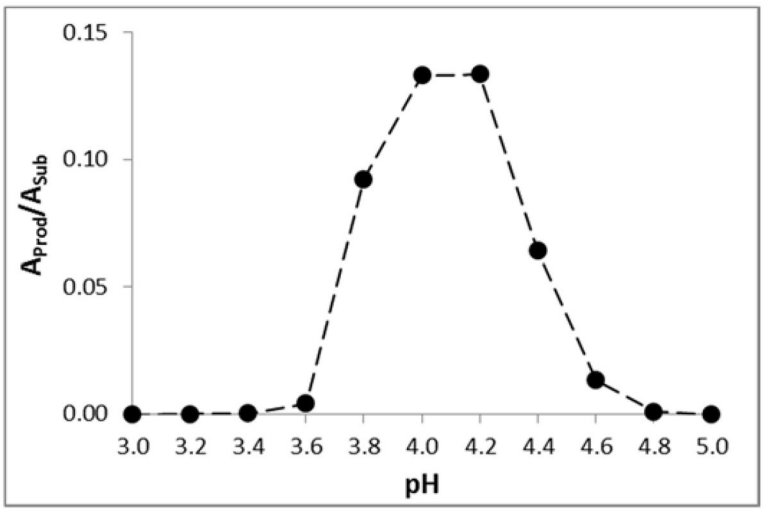


Figure 3.
Measurement of the ricin depurination of RNA14 under different pH conditions.

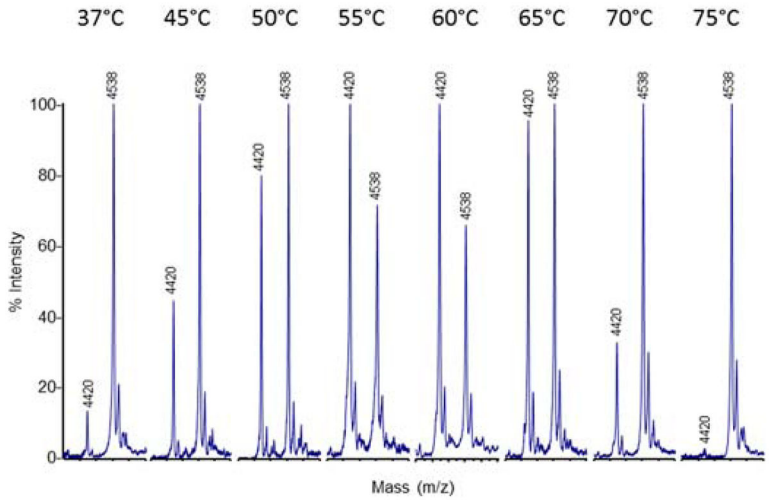
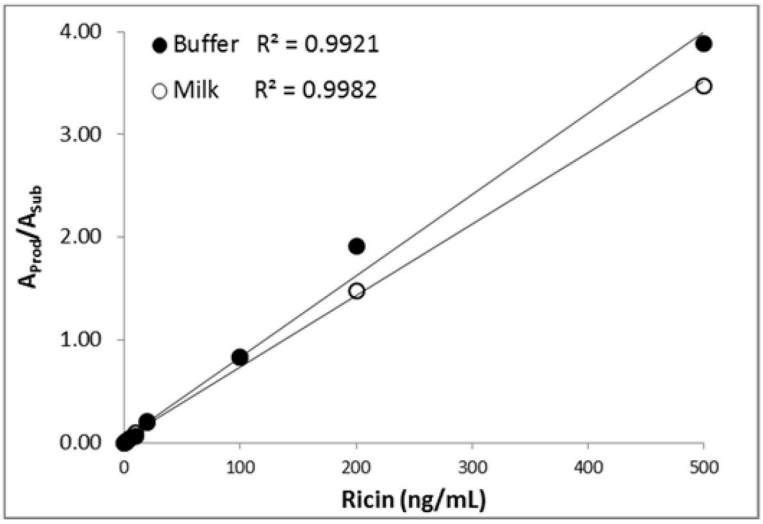


Figure 4.

Hydrolysis of RNA14 substrate by ricin under different incubation temperatures. The peak at 4538 m/z and at 4420 m/z corresponds to unreacted substrate and depurinated product, respectively. Reaction condition: 5 ng ricin, 50μM RNA14 in pH4.0 buffer at various temperatures (37°C– 75°C) for 4 hours.

A.



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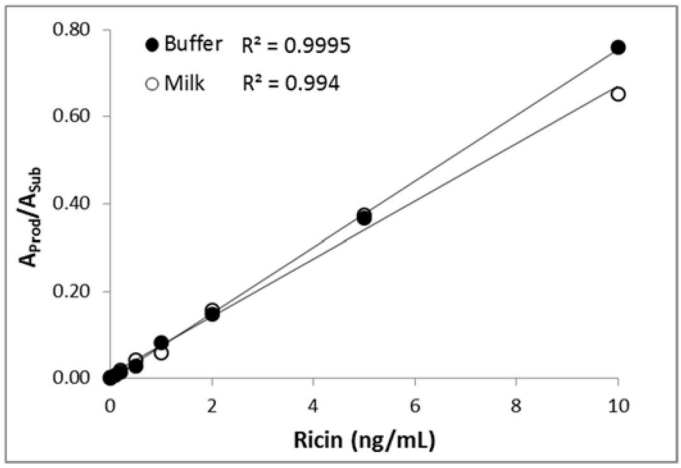

C.

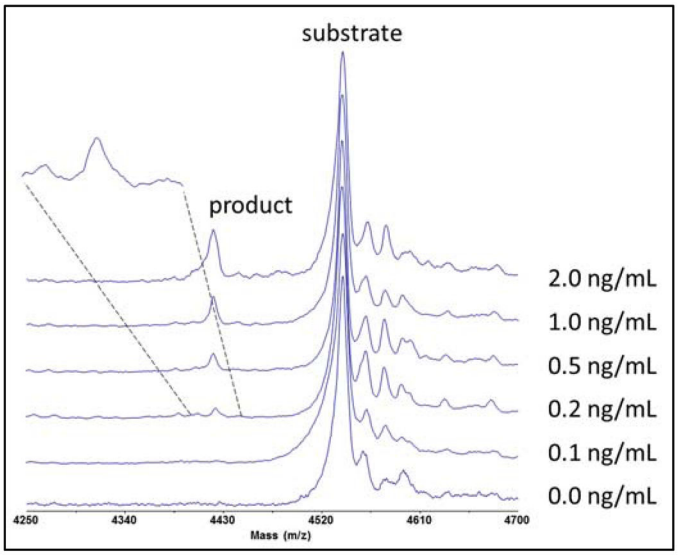

Figure 5.

Detection of the cleavage product from RNA14 by various concentrations of ricin after incubation for 0.5 hour (A) and 4 hours (B). The toxin was spiked in 0.5 mL PBST buffer (●) or 2% milk (○) followed by enrichment with specific antibodies immobilized on magnetic beads. (C). Some typical mass spectra obtained from the aliquots of the ricin activity reaction (55°C, 4 hours).

Table 1

Depurination of various synthetic DNA and RNA substrates by ricin.

Substrate	Sequence	M.W. (Da)	A_{Prod}/A_{Sub} (MALDI-MS)[c]
DNA12	5′-(GCGC**GAGA**GCGC)-3′	3696.4	0.4
RNA14	5′-RNA(CGCGC**GAGA**GCGCG)-3′	4537.8	15.5
$RNA_{Me}14$[a]	5′-RNA_{Me}(CGCGC**GAGA**GCGCG)-3′	4735.6	ND
$RNA_{F}14$[b]	5′-RNA_{F}(CGCGC**GAGA**GCGCG)-3′	4566.6	ND
RNA16-3GA	5′-RNA(CGCGC**GAGAGA**GCGCG)-3′	5212.2	0.3
RNA16-4GA	5′-RNA(CGCGC**GAGAGAGA**GCGCG)-3′	5886.6	ND
RNA16	5′-RNA(GCGCGC**GAGA**GCGCGC)-3′	5188.2	4.7

[a] Consist of 2′-O-methyl-RNA residues.

[b] Consist of 2′-fluoro-RNA residues.

[c] Ratio of the peak areas of the depurinated product (Prod) and unreacted substrate (Sub) acquired by MALDI-MS

Table 2

The peak area ratio of the cleavage product peak (4420 Da) and the nonreacted substrate (4538 Da) detected with different MALDI matrices [a].

No	MALDI matrix composition			A_{prod}/A_{sub}
	3-HPA[b] (M)	Ammonium citrate (mM)	Ammonium tartrate (mM)	
1	0.50	0	10	0.035 ± 0.007
2	0.50	20	10	0.040 ± 0.009
3	0.50	40	10	0.061 ± 0.013
4	0.50	80	10	0.083 ± 0.010
5	0.25	40	10	0.119 ± 0.019
6	0.25	40	5	0.102 ± 0.029
7	0.25	40	2	0.105 ± 0.006
8	0.10	40	10	ND

[a]The cleavage reactions were conducted with 10 ng ricin and 50 M RNA14. The experiments were run in triplicate.

[b]3-HPA represents 3-Hydroxypicolinic acid.

Table 3

Effect of the MALDI spot drying method on the spot-to-spot variation and the peak ratios of product versus substrate (3 spots per sample)

Sample	Toxin (ng)	Vaccum dry		Air dry	
		A_{prod}/A_{sub}	CV (%)	A_{prod}/A_{sub}	CV (%)
A	500	4.54 ± 0.11	2.51	4.75 ± 0.38	8.04
B	500	5.94 ± 0.22	3.75	5.71 ± 1.12	19.62
C	500	5.88 ± 0.24	4.07	6.29 ± 0.69	10.94
E	50	0.84 ± 0.03	3.84	0.97 ± 0.20	20.34
F	50	0.93 ± 0.04	3.83	1.32 ± 0.21	15.92
G	50	0.95 ± 0.02	1.72	1.09 ± 0.36	24.09