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Rapid analysis of ricin using hot acid digestion and MALDI-TOF mass spectrometry

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Abstract

Ricin is a protein toxin of considerable interest in forensics. A novel strategy is reported here for rapid detection of ricin based on microwave-assisted hot acid digestion and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. Ricin samples are subjected to aspartate-selective hydrolysis, and biomarker peptide products are characterized by mass spectrometry. Spectra are obtained using post source decay and searched against a protein database. Several advantages are offered by chemical hydrolysis, relative to enzymatic hydrolysis, notably speed, robustness, and the production of heavier biomarkers. Agglutinin contamination is reliably recognized, as is the disulfide bond strongly characteristic of ricin.

Keywords

agglutinin; curved field reflectron; hot acid digestion; MALDI-TOF; post source decay; protein toxins; ricin

1 | INTRODUCTION

Ricin is produced from the castor bean plant *Ricinus communis* and 1 of the most toxic protein toxins belonging to type 2 ribosome-interacting proteins.¹ Because of its high toxicity, ricin has been categorized as a biological warfare agent and listed in Schedule I of the Chemical Weapons Convention. The lethal dose (LD50) for humans is estimated at 3 and 30 mg/kg kilogram, by inhalation or ingestion respectively.² Historically, ricin has been notoriously linked to the assassination of the Bulgarian journalist Georgi Markov and the most recent “ricin letters” sent to the 44th President of the US Barack Obama.

Ricin has a molecular mass of 62 kDa and comprises 2 chains connected by a disulfide bond. Effective mass spectrometry-based methods have been established that analyze peptide biomarkers using either liquid chromatography coupled to mass spectrometry (LC-MS)^{3–6} or matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass

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SUPPORTING INFORMATION

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spectrometry.⁷ A typical mass spectrometry-based workflow includes ricin enrichment/sample cleanup, protein hydrolysis (peptide production), and mass spectrometry analysis. Enzymatic-based tryptic digestion is the most widely used method for protein hydrolysis and has been successfully coupled to mass spectrometry for the detection of ricin.^{3–7} This method is complex and slow as usually practiced, with protein samples being reduced, and alkylated before a 4 to 16-hour incubation. Several fast tryptic digestion methods have been introduced, including on-target digestion^{8–10} and online digestion,¹¹ albeit with sensitivity reduced by incomplete digestion. Enzymes are bioactive proteins, and controlled temperature and pH are required for their storage as well as for successful use.

To address current limitations in mass spectrometry-based ricin detection methods, a novel strategy is reported here to characterize ricin using hot acid digestion. Microwave-assisted hydrolysis provides residue-specific protein cleavage,^{12–15} and peptide products are characterized by MALDI-TOF mass spectrometry. Post source decay (PSD) analysis in the reflectron mode coupled with online protein database searching was used to increase tolerance to contamination and the specificity of the analysis.

2 | EXPERIMENTAL

2.1 | Safety

Ricin and other protein toxins are dangerous to human health. All of the protocols used in this work have been approved by the University of Maryland Institutional Biosafety Committee. All work with ricin toxin were conducted inside a lab biosafety cabinet (Biosafety Level II). NaOCl (5%) solution with 30-minute contact time was used for ricin inactivation, and waste was autoclaved at 121°C for 1 hour.

2.2 | Materials

Ricin (*communis* agglutinin II) and ricin B chain were obtained commercially from Vector Laboratories (Burlingame, CA). Matrix-assisted laser desorption/ionization-time-of-flight matrix α -cyano-4-hydroxycinnamic acid (CHCA) was purchased from Sigma (St. Louis, MO). Glacial acetic acid, water, trifluoroacetic acid, formic acid, and acetonitrile were purchased from Fisher Chemical (Fair Lawn, NJ).

2.3 | Microwave-assisted hot acid digestion

Microwave-assisted hot acid digestion was conducted in a laboratory microwave (CEM, Matthews, NC). Ricin was prepared in 10 μ L of water, which was diluted to 100 μ L of aqueous 12.5% acetic acid solution. The digestion was conducted at 140°C. Although hydrolysis of ricin was detectable after 3, 5, and 15 minutes, for safety reasons and complete destruction of the protein toxin, ricin samples were routinely hydrolyzed for 15 minutes in this study. The nontoxic ricin B chain was extensively hydrolyzed at 60 seconds. Digested peptides were concentrated and desalted using C18 ZipTips (Millipore, Billerica, MA).

2.4 | MALDI-TOF analysis

α -Cyano-4-hydroxycinnamic acid matrix was prepared in 50% acetonitrile containing 0.1% trifluoroacetic acid as a concentration of 10 mg/mL. Eluted peptides were mixed with 0.5 μ L

CHCA matrix and applied to the MALDI plate. After the samples dried completely, the plate was inserted into the Axima-CFR time-of-flight instrument (Kratos Analytical by Shimadzu Biotech, Manchester, UK). Matrix-assisted laser desorption/ionization-time-of-flight mass spectra were obtained in linear mode with a 337 nm N₂ laser (laser power: 90 arbitrary units), and all spectra were collected as an average of 1000 profiles from 1000 to 5000 Da. For PSD analysis (without CID) product ion spectra were collected in reflectron mode. Precursors were selected with the ion gate set to the biomarker mass $\pm$ 7 Da, and PSD spectra were collected with a sum of 1000 profiles.

2.5 | Negative control experiment

The entire analytical procedure was carried out with ricin being omitted. See Supplementary Figure 1.

2.6 | Data analysis

The online program MS/MS Ions Search (Matrix Science, Boston, MA) was used to analyze ion fragment ions collected from PSD. Mass and intensity data collected from PSD analysis were converted into the MGF file format and submitted for analysis. “NCBIprot” protein database was used, and “green plants” was selected for taxonomy. Enzyme was defined as “Formic_acid,” and 3 missed cleavages were allowed. Dehydration and oxidation were included in the variable modifications.^{13,14} The precursor mass tolerance was set to 1 Da, and fragmentation tolerance was 0.6 Da. Mass type was defined as “average,” and the instrument type defined as “MALDI-TOF-PSD.”

2.7 | Bottom-up proteomics

Protein hydrolysis patterns and peptide identities were confirmed using a bottom-up proteomic strategy, with an Ultimate 3000 Nano HPLC system (Dionex, Sunnyvale CA) interfaced by nanoelectrospray to an orbitrap Fusion Lumos instrument (Thermo Fisher Scientific, San Jose CA). The column was C-18 (Dionex), and the gradient was from 5% to 55% solvent B (97.5% acetonitrile, 2.5% water, and 0.1% formic acid) in 90 minutes. Data analysis was provided by Proteome Discoverer v.2.1.

3 | RESULTS AND DISCUSSION

3.1 | Characterization of peptides produced from ricin by microwave-assisted hot acid digestion

Acid-catalyzed proteolysis has been shown to cleave with high specificity on the carboxyl and amino bonds of aspartic acid residues in proteins.^{12–15} After microwave-assisted hot acid digestion of 100 ng commercial ricin, 44 peptide peaks were observed in the mass range 1000 to 5000 Da with signal to noise ratios >10 (Figure 1 and Supplementary Table 1). No peaks are observed above m/z 1000 in mass spectra of the matrix or the negative control (Supplementary Figure 1). Peptides in this high mass range were identified in an independent bottom-up proteomic analysis by LC-tandem mass spectrometry (Section 2) and are presented in Supplementary Table 1. The peptides mapped in the MALDI-TOF spectrum provide 75% of the ricin sequence, and all these peptides are cleaved at either the amino or the carboxyl bonds of aspartate residues, confirming the excellent selectivity of hot

acid cleavage for aspartyl residues (Supplementary Table 1). The 2 most abundant peaks in Figure 1 were characterized as the protonated carboxyl-terminus peptide PSLKQIILYPLHG (1479.6 Da) and its +115 Da analog (1594.9 Da), which carries an additional aspartic acid residue. The only 2 peptides predicted but not observed are amino acids 160 to 235 and amino acids 409 to 490. The masses of both of these peptides exceed 5000 Da and thus were not included in the data collection. (Residues are conventionally numbered as in the unprocessed gene product.)

Ricin comprises 2 protein chains linked by a disulfide bond between cysteines 294 and 318. In this study, the mass of peptide 280 to 319 in the MALDI spectrum indicates that it contains a disulfide bond as well as peptides from both chains ($M + H$ 4287.8 Da, Figure 1 and Supplementary Table 1). This observation is consistent with earlier observations that disulfide bonds are not reduced during hot acid without adding DTT.^{13,14} Important from the view of ricin analysis, the mass of this linked peptide provides a unique ricin biomarker formed with hot acid treatment.

Ricinus communis agglutinin is a protein that is often isolated as a contaminant of ricin.^{6,16,17} Eight peptides were identified in the MALDI spectrum as biomarkers of agglutinin in the commercial ricin sample used here (Supplementary Table 1). It is suggested that detection of agglutinin could provide additional forensic information on samples of ricin.

Characterization of acid hydrolysis products provides a basis for detection of relatively pure ricin by peptide maps or for selection of peptide biomarkers for a more rigorous identification based on PSD.

3.2 | Identification of ricin using PSD analysis of peptide biomarkers

Spectra of fragment ions formed by PSD are expected to provide reliable identifications of contaminated ricin in a rapid timeframe.^{18–20} Selection of biomarkers for PSD can also be automated in a robust TOF configuration. Representative MALDI-PSD spectra acquired from protonated peptide precursors produced from a 5 ng sample are shown in Figure 2. The precursor ions selected are formed from ricin A chain, ricin B chain, and agglutinin. Extended *b* and *y* ion series are observed for both peptides. Toward the objective of rapidly identifying peptides, and accordingly ricin, fragment ion spectra were subjected to database searches using MASCOT (Table 1).

In another experiment, 8 of the ricin-specific peptides from a 100 ng ricin sample were manually isolated for sequential PSD. The overall mass range was narrowed to between 1000 and 2000 Da, and the peptides selected are known from our first experiment (Supplementary Table 1) to be unique to ricin within the *SwissProt* database. All 8 peptides were identified and assigned to ricin (Table 1) with statistical significance (*E* value <0.05). The results demonstrate that PSD analysis of characteristic peptides obtained by acid cleavage can provide unambiguous identification of ricin.

The hot acid method provides several advantages over trypsin digestion. Most importantly, hydrolysis can be driven to completion in minutes. No additional step is required to denature

the sample. Coverage is comparable or higher.^{7,10} Autolysis does not contribute interfering peptides. No restrictions exist on temperature and pH for storage, portability or use.

On average, protein cleavage on either side of aspartate residues produces longer peptides than cleavage at the carboxyl termini of arginine and lysine.¹⁴ Mature ricin contains 26 Asp residues which lead to 27 cleavage products (theoretically) of which only 6 have masses below 1000 Da. In contrast, ricin contains 45 Lys and Arg residues, which generate 46 theoretical tryptic products, including 21 with masses below 1000 Da. Short peptides overlap with, and are often suppressed by, matrix clusters. Longer peptides usually provide more significant links to their parent protein. On the other hand, 1 strength of tryptic digestion is the more even distribution of chargeable basic residues across the product mixture.

4 | CONCLUSIONS

In this study, we have demonstrated a method using hot acid protein hydrolysis for the rapid characterization of ricin with MALDI-TOF mass spectrometry. Of particular interest, our method identifies the disulfide bond uniquely characteristic of ricin and also provides reliable identification of agglutinin peptides of forensic interest. The method provides specificity, efficiency, speed, robustness, and the potential for automation. We have also applied the strategy of hot acid digestion and MALDI-TOF successfully (data not shown) to the protein toxins abrin, botulinum toxin type A, and *Staphylococcal* enterotoxin B.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Ricin, hot acid (140°C for 15 minutes). MALDI-TOF mass spectrum of peptides produced from a sample containing 100 ng of commercial ricin m/z 1000 in mass spectra of the matrix or the negative control (Supplementary Figure 1). Peptides in this high mass range were identified in an independent bottom-up proteomic analysis by LC-tandem mass spectrometry (Section 2) and are presented in Supplementary Table 1. The peptides mapped in the MALDI-TOF spectrum provide 75% of the ricin sequence, and all these peptides are cleaved at either the amino or the carboxyl bonds of aspartate residues, confirming the excellent selectivity of hot acid cleavage for aspartyl residues (Supplementary Table 1). The 2 most abundant peaks in Figure 1 were characterized as the protonated carboxylterminus peptide PSLKQIILYPLHG (1479.6 Da) and its +115 Da analog (1594.9 Da), which carries an additional aspartic acid residue.

FIGURE 2.

Post source decay, PSD spectra of 5 ng ricin sample with hot acid. Insets show the precursor ions selected using the ion gate. A. Product ion distribution from precursor ions of mass 1537.6 Da (Ricin A chain: VQNRYTFAFGGNY). B. Product ion distribution from precursor ions of mass 1594.9 Da (Ricin B chain: PSLKQIILYPLHG). C. Product ion distribution from precursor ions of mass 2713.2 Da (Agglutinin: PSLKQIIVHPFHGNNLNQIWLPLF)[dummy]

TABLE 1Peptides identified from PSD spectra and *E* values from MASCOT

Peptide Sequence (Ricin)	Position (Amino Acids)	(MH+, Da)	<i>E</i> Value (MASCOT Analysis)
ATRWQIWD	401–408	1076.2	0.045
PNQIWLPLF	568–576	1128.4	0.0023
ANQLWTLKRD	359–368	1245.4	0.014
PSLKQIILYPLHG	554–566	1479.8	4.50E–08
VQNRYTFAFGGNY	146–158	1537.7	0.045
PSLKQIILYPLHGD	554–567	1594.9	9.70E–08
GTILNLYSGLVLDVRAS	536–552	1791.2	1.20E–05
GTILNLYSGLVLDVRASD	636–553	1907.2	2.20E–07