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APPLICATION NOTE

Rapid Detection of Ricin in Serum Based on Cu-Chelated Magnetic Beads Using Mass Spectrometry

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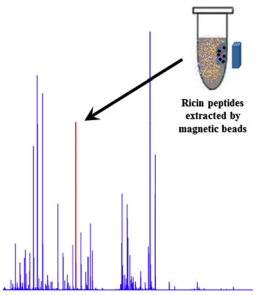

Abstract. The protein toxin ricin obtained from castor bean plant (Ricinus communis) seeds is a potent biological warfare agent due to its ease of availability and acute toxicity. In this study, we demonstrated a rapid and simple method to detect ricin in serum in vitro. The ricin was mixed with serum and digested by trypsin, then all the peptides were efficiently extracted using Cu-chelated magnetic beads and were detected with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The specific ricin peptides were identified by Nanoscale Ultra Performance liquid chromatography coupled to tandem mass spectrometry according to their sequences. The assay required 2.5 hours, and a characteristic peptide could be detected down to 4 ng/μl and used as a biomarker to detect ricin in serum. The high sensitivity and simplicity of the procedure makes it valuable in clinical practice.

Keywords: Ricin, Cu-Magbeads, Mass spectrometry, Rapid detection

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Introduction

Ricin is a naturally occurring toxin produced by the seeds of the castor bean plant [1, 2]. It is among the deadliest known poisons with an LD50 in humans of 5-10 μg/kg through inhalation or 350-700 μg for a 70 kg person [3, 4]. The ease of production of the toxin from the castor bean seeds and the accessibility of the plants in several countries have led ricin to be considered a biological weapons by the US Centers for Disease Control and Prevention (CDC) [3, 5, 6].

Ricin is a protein with a molecular weight of approximately 65 kDa [3, 7]. It comprises of an A-chain disulfide bonded to a B chain [8]. The ricin toxin A chain (RTA) is a ribosome inactivating protein (RIP) that inhibits protein synthesis in mammalian cells. The ricin toxin B chain (RTB) is a lectin that binds to galactose residues on the surface of cells [1].

Ricin A chain exhibits RNA N-glycosidase activity that hydrolyses a specific adenine residue present at the 4324 position from a highly conserved loop region of 28S rRNA. This activity prevents the formation of the critical stem loop configuration to which the elongation factor 2 is known to bind during translation. This results in complete inhibition of cellular translation and cell death [9–11].

Ricin is dangerous not only because of its extremely strong toxicity, but also because it is water soluble and stable under heat and a wide pH range [12]. Sensitive and selective analytical methods are thus needed to rapidly detect ricin in complex systems such as serum, food and clinical samples [3]. The most common methods of detection are immunoaffinity assays due to their sensitivity and utility under aqueous conditions [13, 14]. However, when the ricin dissolves in serum, these assays are not always unambiguous because false positives may result from cross reactivity with other molecules containing similar binding motifs.

Recently, a detection strategy based on serum peptide profiling by magnetic beads has been developed with a distinct advantage for this situation [15]. The ability of the magnetic beads to separate and enrich peptides from serum makes it possible to detect peptide toxins [16]. Along with mass spectrometry, it is rapid, selective, and sensitive in detecting various peptides and small nucleic acids by using magnetic beads and therefore should provide a sensitive method for analysis [17, 18]. The sensitivity and resolution of new mass spectrometry allows for the detection of ricin.

It takes a long turnaround time for existing methods used antibodies and sensors, which is complicated operations and poor reproducibility [19]. And ELISA had a potential use of blood samples to identify cases of ricin poisoning [7]. In this study, we

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used mass spectrometry to analyze ricin instead of traditional detection methods. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is an effective and fast technique to accurately detect ricin peptides [20, 21]. It also can be used to screen biomarkers in complex systems including serum [16, 22]. It plays a key role in the toxin's diagnosis and therapy [23, 24]. Nanoscale Ultra Performance liquid chromatography coupled to tandem mass spectrometry (NanoUPLC-MS/MS) was used for determination of ricin in solution because of its high sensitivity and the acquisition of reliable data from independent protein digests. The ricin tryptic peptides were thus accurately identified by data-dependent analysis [25].

In this study, we establish a method that combines Cu-chelated magnetic beads (Cu-Magbeads) with mass spectrometry for the rapid and accurate detection of ricin in serum. A mixture of ricin and serum was digested by trypsin and treated with Cu-Magbeads. The final extractions are analyzed by MALDI-TOF-MS, and the characteristic peptides are then identified by NanoUPLC-MS/MS. Collectively, this protocol provides a sensitive and selective analysis of ricin isolated from serum.

Materials and Methods

Materials

Trypsin was obtained from Roche (Germany). Matrix α-cyano-4-hydroxycinnamic acid (4HCCA) was purchased from Bruker Daltonics GmbH (Bremen, Germany). Acetonitrile (high-performance liquid chromatography grade) and trifluoroacetic acid (sequence grade) were purchased from Fluka (Buchs, Switzerland). Water was prepared by a Milli-Q system (Millipore). Purified water was purchased from Merck (Germany).

Serum Samples

Blood samples from healthy volunteers were collected from the 301 General Hospital using informed consent. 600 µl of serum were divided into three aliquots and stored at –80 °C until further use.

Ricin-Spiked Serum Samples

Ricin is very toxic and requires appropriate safety measures. All toxins were handled within a class 2 biosafety cabinet equipped with high energy particulate air (HEPA) filters. For detection tests, serum samples were mixed with 1 mg/ml of ricin. For sensitivity testing, the final concentration of ricin was 20, 10, 5, 4, 2.5, and 2 ng/µl.

Denaturation and Tryptic Digestion

Ricin-spiked serum samples were incubated with 100 mM dithiothreitol (DTT) at 60 °C for 1 h. After cooling to the room temperature, the samples were mixed with 55 mM iodoacetamide and placed at room temperature for 30 mins. Then, at a ratio of 1:50, enzyme to protein substrate, the mixtures were subjected to digest using trypsin for overnight at 37 °C in an incubator [16].

Sample Preparation for MS Analysis

The following protocol was adopted for the analysis of the serum samples. The ricin solution and the mixture of ricin and serum were both digested using trypsin. Twenty microliters of the Cu-Magbeads was mixed with 50 µl of binding solution in a 200 µl tube. Then the beads were fixed at the tube wall with a magnetic separator, and the supernatant was carefully discarded. This procedure was repeated twice. The Cu-Magbeads were resuspended in 20 µl of binding solution, and 10 µl of serum was added and mixed carefully. The mixture was kept at room temperature for 10 min. Then the Cu-Magbeads were filtered from the solution and washed three times with washing solution. The analytes were eluted from the Cu-Magbeads with eluant. Finally, the eluant was splited into 2 portions centrifuged for MALDI-TOF-MS and NanoUPLC-MS/MS analysis respectively.

Mass Spectrometry Analysis

MALDI-TOF-MS (UltraFlexIII, Bruker Daltonics) and an Acquity NanoUPLC system (Waters Corp.) with a Q-TOF (SYNAPTTM G2S, Waters) were used for the detection of peptides. The procedure and parameters of MALDI-TOF-MS and NanoUPLC-MS/MS were described detailedly in the Supplementary Information.

Results and Discussion

Characterization of Ricin

To identify ricin, we designed several steps: 1 mg/ml ricin in aqueous solution was mixed with an equal volume of sinapinic acid. MALDI-TOF-MS was then used to detect the molecular weight of the mixture, and two distinct peaks were obtained: a single charge (*m/z* 63,528) and a double charge (*m/z* 31,733) (Figure 1A). The peptide mass fingerprinting of ricin containing the A chain and B chain were acquired using the MALDI-TOF-MS (Figure 1B). Then, NanoUPLC-MS/MS was used to identify the basic sequence of ricin, including the Total Ion Chromatography (TIC) peaks of the total peptides and the sequence identification of the ricin peptides (Figure 1C, D, and E). We detected ten peptides that had the same mass-to-charge ratio as the theoretical enzymatic digests. The sequences of these ten peptides were identified with NanoUPLC-MS/MS (Supplemental figure 1). 4 of the 10 peptide sequences of ricin listed in Supplemental Table 1 are common with agglutinin.

Evaluation of the Cu-Magbeads Treatment

To detect the peptide digests of ricin treated with Cu-Magbeads, 10 µg of the tryptic digest was treated with 10 µl of the Cu-Magbeads. The extracts were then eluted with 20 µl of elution buffer. The 1 µl of the final extract was detected by MALDI-TOF-MS from 500 Da to 5000 Da [25]. According to the theoretical mass fingerprints of ricin peptides, we gained 10 peaks with S/N ratios greater than 10 (Supplemental Figure 2).

The serum peptides could not be observed directly [26]. This may be due to the salts, buffers or other high abundant species in

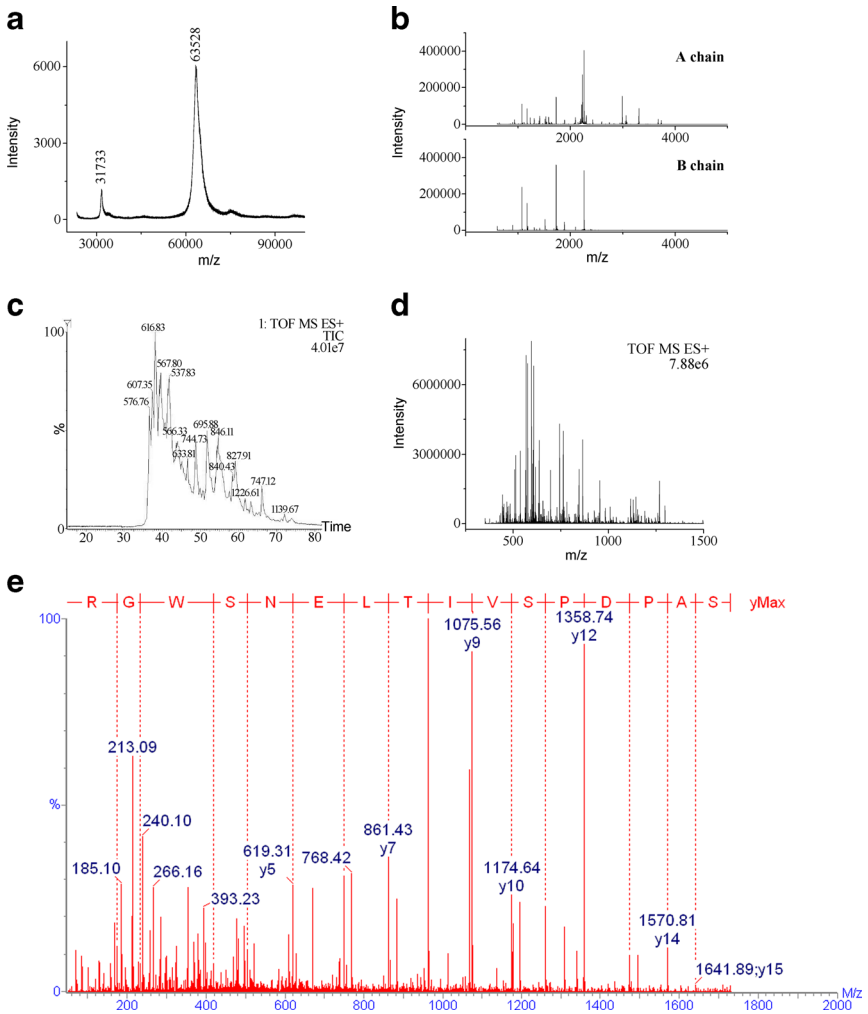

Figure 1. Identification of ricin. The molecular weight of ricin (**a**); The in-gel tryptic peptide mass finger prints of the two chains of ricin (**b**); The TIC of ricin peptides using nanoUPLC-MS/MS (**c**); The total peptide peaks using nanoUPLC-MS/MS (**d**); The identification of sequence of 1728.8551 Da (**e**)

the serum. These factors may contribute to inhibition or disappearance of the MS signal [27]. In subsequent experiment, we observed sixteen peaks of ricin digest with MALDI-TOF-MS, but only ten peaks were observed after treating with the Cu-Magbeads, which might be because that histidine, tryptophan, or cysteine residues of the small peptides can bind to the Cu-Magbeads [28]. Our study suggested that Cu-Magbeads was effective at concentrating low molecular weight peptides from the ricin digest.

Reproducibility

Previous studies have shown that magnetic beads can be effective at separating and enriching characteristic peptides of ricin. For the reproducibility experiment of the Cu-Magbeads treatment, serum was digested by trypsin and treated with Cu-Magbeads. We performed six replicate analyses using MALDI-TOF-MS (Supplemental Figure 3). This data is listed in Supplemental Table 2. The statistical results show that the CV of mass-to-charge was 0.0052%, and the CV of the relative intensity was 12.5%. These results show that the method had an acceptable precision.

Sensitivity

To understand the extraction effects of the Cu-Magbeads at extremely low concentrations of ricin, we used different amounts of ricin tryptic digestion treated with Cu-Magbeads. The final concentrations were from 20 ng/µl to 2 ng/µl (Supplemental Figure 3A). We detected a stable peak with MALDI-TOF-MS (1728.8551 Da) that was sensitive down to 4 ng/µl (Supplemental Figure 3B). The theoretical sequence was identified to be SAPDPSVITLENSWGR by NanoUPLC-MS/MS.

Detection of Ricin in Serum

Our results show that the Cu-Magbeads can efficiently isolate and enrich low abundant peptides. We used the Cu-Magbeads coupled with MALDI-TOF-MS to detect the 1728.8551 Da peptide in serum. To demonstrate the efficacy of the method, ricin was mixed into serum and digested by trypsin at a final concentration of 4 ng/µl (data not shown). Ten microliter sample aliquots were mixed with 10 µl of activated Cu-Magbeads. After washing and eluting, the ricin peptides and

other low molecular weight serum peptides were collected [29–31]. The eluents were assayed by MALDI-TOF-MS (Supplemental Figure 4A). We identified the sequence of this peptide to be SAPDPSVITLENSWGR (Supplemental Figure 4B).

Conclusions

Ricin is a potential bioterrorist weapon, and the presence of ricin should be confirmed using complementary analytical methods. Immunoassays are highly sensitive but have many limitations [13, 14]. Mass spectrometry is specific, but rather insensitive [16, 21, 22]. Here we report a simple and rapid method combining Cu-Magbeads with MALDI-TOF-MS to detect tryptic peptides of ricin in serum, and use NanoUPLC-MS/MS to identify the sequences of the peptides. This approach is highly sensitive via the Cu-Magbeads approach and specific due to the use of mass spectrometry. This method has potential use to identify toxic agents for further clinical research with other applications in metabolomics.

Acknowledgments

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