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ARTICLE *in* ANALYTICAL CHEMISTRY · APRIL 2005

Impact Factor: 5.64 · DOI: 10.1021/ac048756u · Source: PubMed

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Articles

Forensic Identification of Neat Ricin and of Ricin from Crude Castor Bean Extracts by Mass Spectrometry

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The protein toxin ricin, which originates from the seeds of *Ricinus communis* plants, has been the subject of increased interest, due to its potential terrorist use. Exceptionally, this toxin is also subject to the Chemical Weapons Convention. In this paper, it is shown that mass spectrometry can be used to unambiguously verify the presence of ricin in crude toxin preparations. It is demonstrated that MALDI MS can be used for screening, either by direct analysis or by trypsin digestion and peptide mapping. Purified ricin from several varieties of *R. communis* was characterized by LC−ES MS(/MS). A crude ricin preparation from a single bean was similarly characterized. An LC method was set up with product ion MS/MS detection of selected marker peptides specific for ricin: T5, T7, T11, T12, and T13 from the A-chain and T3, T5, T14, T19, and T20 from the B-chain. This method was then used to unambiguously identify ricin in a crude preparation of ricin. The MALDI MS molecular weight analysis and the marker peptides LC−ES MS/MS analysis give a forensic level of identification of ricin when combined with activity testing.

Among the midspectrum agents, toxic compounds of biological origin, ricin takes an exceptional place, because it is the only protein listed under the Chemical Weapons Convention.[1] Ricin is produced in *Ricinus communis* seeds, otherwise known as castor beans. These beans are widely used in the production of castor oil, which end product is typically applied as a hydraulic fluid, in paints, motor oil, and other industrial applications. Ricin makes up 1−5% of the pulp that remains after processing of the beans for oil production. The annual world production of castor beans has steadily been growing and has now reached a level in excess of 1 million tons.[2] This large production implies that the process waste accounts for a huge possible source of ricin. The waste is either dumped, with subsequent biodegradation of ricin, or treated by heating with concomitant irreversible inactivation of ricin; inactivation is recommended by the Organization for the Prohibition of Chemical Weapons (OPCW).[3] The fact that ricin can be extracted and purified relatively easily from the bean pulp makes the compound a potential threat agent for military or terrorist use.

Ricin is highly toxic, with a typical mouse LD_{50} in the order of 2 μg/kg of body weight, intraperitoneally;[4] unofficial sources give a human LD_{50} of 3−30 μg/kg by inhalation or ingestion, respectively. At the assassination of the Bulgarian journalist Georgi Markov, by injection of ricin, the dose administered was estimated to have been 0.5 mg.[5] The general mechanism of action of ricin is known at the molecular level. A ricin molecule consists of an A- and a B-chain, linked by a disulfide bridge. Both chains have a molecular weight of ∼32 000. The B-chain is responsible for binding to cells and internalization of the toxin in those cells, although autonomous binding and uptake of purified A-chain by specific cells may occur.[6] The A-chain exerts the real toxic action, as an RNA N-glycosidase, in that it catalytically inactivates ribosomes, the protein manufacturing organelles inside a cell. This RNA N-glycosidase activity is known to occur with high specificity by depurination of adenosine at position 4324 of eukaryotic 28 S ribosomal RNA.[7,8] This ribosomal RNA cleavage disables the

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10.1021/ac048756u CCC: $30.25
Published on Web 02/04/2005

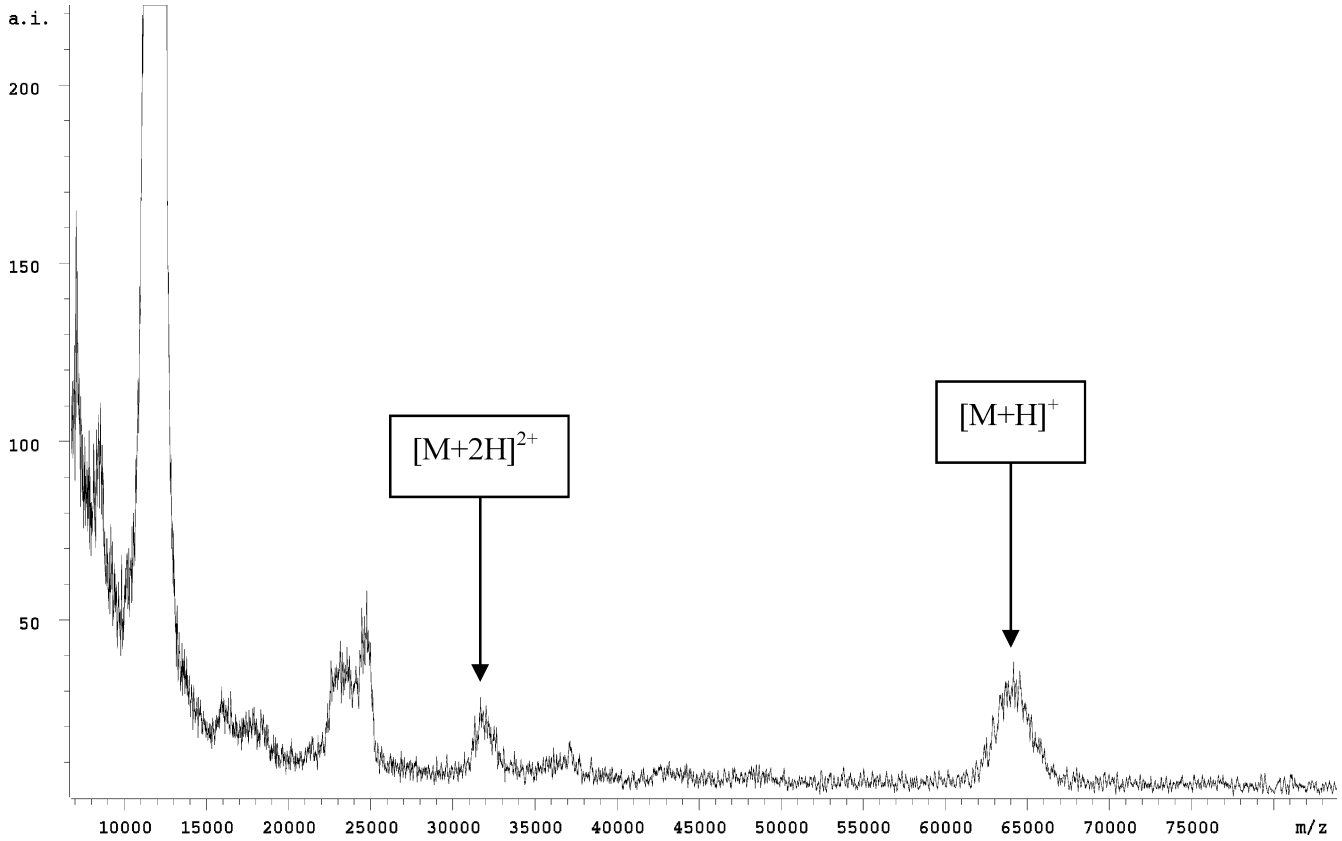

Figure 1. MALDI mass spectrum of intact crude ricin.

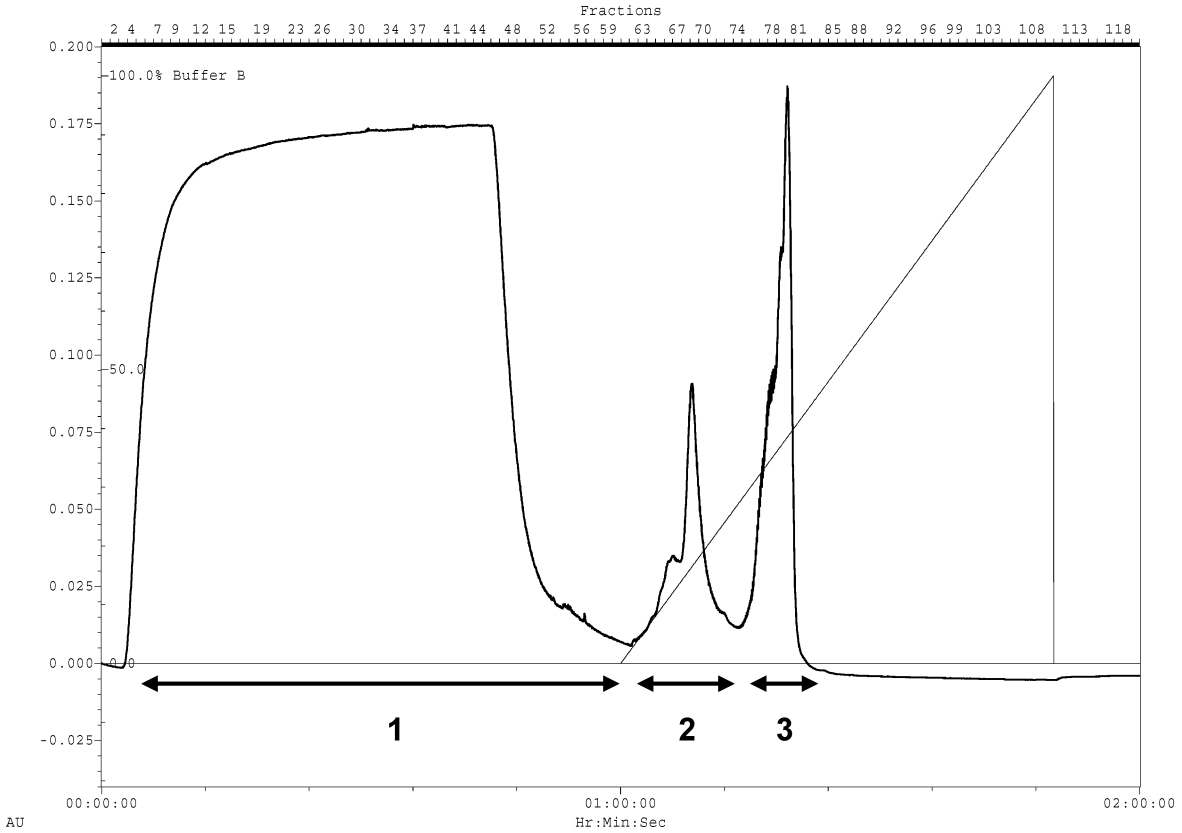

Figure 2. UV chromatogram and gradient plot of the cation-exchange fractionation of affinity-purified ricin from *R. communis carmencita.* At the initial gradient conditions, part of the material was not retained by the column and that eluted as the broad peak. 1. A gradient in Tris-HCl (pH 7.7) from 0 to 500 mM NaCl was started at 11 min and two peaks, 2 and 3, were collected. The identity of the chromatographic peaks was determined by trypsin digestion and LC−ES MS and MS/MS. Peak 1 was shown to be ricin D, while peaks 2 and 3 were ricin E.

Table 1. Comparison of Two Different Amino Acid Sequences of Ricin Chain A (Rows 1 and 2) with That of RCA (Row 3)[a]

Ricin[1]	IFPK**QYPIIN** **FTTAGATVQS** **YTNFIR**AVR**G** **RLTTGADVR**H EIPVLPNR**VG**	50
Ricin[2]	IFPKQYPIIN FTTAGATVQS YTNFIRAVRG RLTTGADVRH EIPVLPNRVG	50
RCA[3]	IFPKQYPIIN FTTADATVES YTNFIRAVRS HLTTGADVRH EIPVLPNRVG	50
	********** **** *** * ********* ********* **********	
Ricin[1]	**LPINQR**F**ILV** **ELSNHAELSV** **TLALDVTNAY** **VVGYRAGNSA** **YFFHPDNQED**	100
Ricin[2]	LPINQRFILV ELQNHAELSV TLALSVTNAY VVGYRAGNSA YFFHPDNQED	100
RCA[3]	LPISQRFILV ELSNHAELSV TLALDVTNAY VVGCRAGNSA YFFHPDNQED	100
	*** ****** ** ******* **** ***** *** ****** **********	
Ricin[1]	**AEAITHLFTD** **VQNRYTFAFG** **GNYDRLEQLA** **GNLRENIELG** **NGPLEEAISA**	150
Ricin[2]	AEAITHLFTD VQNRYTFAFG GNYDRLEQLA GNLRENIELG NGPLEEAISA	150
RCA[3]	AEAITHLFTD VQNSFTFAFG GNYDRLEQLG G-LRENIELG TGPLEDAISA	149
	********** *** ***** ********* * ******** **** ****	
Ricin[1]	**LYYYSTGGTQ** **LPTLARSFII** **CIQMISEAAR** **FQYIEGEMR**T RIRYNRR**SAP**	200
Ricin[2]	LYYYSTGGTQ LPTLARSFII CIQMISEAAR FQYIEGEMRT RIRYNRRSAP	200
RCA[3]	LYYYSTCGTQ IPTLARSFMV CIQMISEAAR FQYIEGEMRT RIRYNRRSAP	199
	****** *** ******* ********** ********** **********	
Ricin[1]	DPSVITLENS WGRLSTAIQE SNQGAFASPI QLQRRNGSK**F** **SVYDVSILIP**	250
Ricin[2]	DPSVITLENS WGRLSTAIQE SNQGAFASPI QLQR-DGSKF SVYDVSILLP	249
RCA[3]	DPSVITLENS WGRLSTAIQE SNQGAFASPI QLQRRNGSKF NVYDVSILIP	249
	********** ********** ********** **** **** ******* *	
Ricin[1]	**IIALMVYR**CA PPPSSQF	267
Ricin[2]	IIA-MVYRCA PPPSSQF	265
RCA[3]	IIALMVYRCA PPPSSQF	266
	*** ****** *******	

[1] Ricin D precursor A-chain from Swissprot [31] (GI:132567);

[2] Ricin D A-chain [29] (GI:223026);

[3] A-chain of RCA precursor from Swissprot [34] (GI:113504).

[a] Identical amino acids are marked by an asterisk; the underlined sequence was confirmed by LC−ES MS/MS, whereas the boldface type sequence indicates possible marker peptides for ricin (trypsin digest peptides with sequence differences compared to RCA).

elongation factor 2 binding essential to protein synthesis[9] and, thus, stops protein production. This cytotoxic action eventually causes cell death, a capability that promoted many investigations into selective killing of cancer cells by ricin A-chain-based artificial proteins (see, for example, ref 10). The catalytic nature of the ribosome incapacitation implies that one molecule of ricin can stop the activity of many ribosomes; indeed, one A-chain molecule is sufficient to cleave all ribosomes in a single cell and thus kill that cell.[11] The catalytic nature of the toxic process is the main reason for a low lethal dose, and internalization of the toxin into a cell can be seen as the toxicity-determining step.

Detection and identification of ricin has been attempted in many ways. Most detection and identification methods are typical bioassays: activity testing with ribosomes (see, for example, refs 12−14), assay of adenine release from 3H-adenosine-labeled DNA,[15] and antibody reaction (for example, in an enzyme-linked immunosorbent assay).[16,17] These assays are not specific to ricin, because other lectins may also cleave ribosomes in the same way[12] or antibodies may suffer from cross-reactivity. However, only a few reports have been published on chemical instrumental methods of analysis, with a generally higher specificity. Ricin was characterized by electrospray mass spectrometry, capillary electrophoresis, and surface plasmon resonance (using lectin adsorption).[18] The mass spectrometry characterization encompassed electrospraying of intact ricin molecules. Both mass spectrometry and electrophoresis were used to reveal the heterogeneity of ricin from different cultivars of *R. communis* seeds. This heterogeneity is generally not considered in detection or toxicity studies, but mass spectrometric analysis implicitly produces a variety of molecular weights for the separate ricin iso- and glycoforms. The presence of this biovariation makes the position of ricin on the

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Table 2. Comparison of the Amino Acid Sequences of the B-Chain of Ricin Isoforms D and E with RCA[a]

ricin D[1]	ADVCMDPEPI VRIVGRNGLC VDVRDGRFHN GNAIQLWPCK SNTDANQLWT	50
ricin E[2]	ADVCMDPEPI VRIVGRNGLC VDVRDGRFHN GNAIQLWPCK SNTDANQLWT	50
RCA[3]	ADVCMDPEPI VRIVGRNGLC VDVTGEEFFD GNPIQLWPCK SNTDWNQLWT	50
	********** ********** *** * ** ******* **** *****	
ricin D[1]	LKRDNTIRSN GKCLTTYGYS PGVYVMIYDC NTAATDATRW QIWDNGTIIN	100
ricin E[2]	LKRDNTIRSN GKCLTTYGYP SGVYVMIYDC NTAATDATRW QIWDNGTIIN	100
RCA[3]	LRKDSTIRSN GKCLTISKSS PRQQVVIYNC STATVGATRW QIWDNRTIIN	100
	* * ***** ***** * ** * ** **** ***** ****	
ricin D[1]	PRSSLVLAAT SGNSGTTLTV QTNIYAVSQG WLPTNNTQPF VTTIVGLYGL	150
ricin E[2]	PRSSLVLAAT SGNSGTTLTV QTNIYAVSQG WLPTNNTQPF VTTIVGLYGM	150
RCA[3]	PRSGLVLAAT SGNSGTKLTV QTNIYAVSQG WLPTNNTQPF VTTIVGLYGM	150
	*** ****** ****** *** ********** ********** *********	
ricin D[1]	CLQANSGQVW IEDCSSEKAE QQWALYADGS IRPQQNRDNC LTSDSNIRET	200
ricin E[2]	CLQANSGKVW LEDCTSEKAE QQWALYADGS IRPQQNRDNC LTTDANIKGT	200
RCA[3]	CLQANSGKVW LEDCTSEKAE QQWALYADGS IRPQQNRDNC LTTDANIKGT	200
	******* ** *** ***** ********** ********** ** * ** *	
ricin D[1]	VVKILSCGPA SSGQRWMFKN DGTILNLYSG LVLDVRASDP SLKQIILYPL	250
ricin E[2]	VVKILSCGPA SSGQRWMFKN DGTILNLYNG LVLDVRRSDP SLKQIIVHPF	250
RCA[3]	VVKILSCGPA SSGQRWMFKN DGTILNLYNG LVLDVRRSDP SLKQIIVHPF	250
	********** ********** ******** * ****** *** ****** *	
ricin D[1]	HGDPNQIWLP LF	262
ricin E[2]	HGNLNQIWLP LF	262
RCA[3]	HGNLNQIWLP LF	262
	** ****** **	

1 Ricin-D precursor B-chain from SwissProt [31] (GI:132567);

2 ricin E B-chain [25] (GI:225419);

3 RCA precursor B-chain from SwissProt [34] (GI:113504).

[a] The underlined ricin D sequence was confirmed by LC−ES MS/MS and boldface type sequence indicates possible marker peptides for ricin (trypsin digest peptides with sequence differences compared to RCA).

CWC schedule lists quite strange, because all other CWC scheduled compounds have well-defined, unequivocal molecular structures. The successful application of MALDI MS to molecular weight determination of isoforms of ricin A-chain, in the characterization of a purification process, was recently reported.[19] In the same year, it was argued that "presumptive" forensic identification of ricin can be based on observation of the molecular weight and the trypsin digest peptide map from matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) and the additional observation of ricinin by gas chromatography/mass spectrometry or by liquid chromatography−electrospray mass spectrometry (LC−ES MS).[20] Here, ricinin provides indirect evidence, because it is a marker compound that presumably only occurs in ricin beans. However, this kind of evidence is not irrefutable, because peptide mapping is prone to interference and hence requires careful purification to separate ricin from RCA. In general, protein sequence evidence is required for adequate identification (see, for example, ref 21). Thus, the chemical analysis methods reported so far provide only "provisional identification", because they do not address the core of structural information comprised in the peptide backbone of the toxin.

In the line of our ongoing investigations on identification of midspectrum agent toxins (see, for example, ref 22), we presently report on the mass spectrometric analysis of ricin at the amino acid and glycan sequence level. In the course of this study, it turned out that the information in ricin glycopeptides can also be addressed by mass spectrometry. The relevant glycopeptides MS will be dealt with in a separate paper.

MATERIALS AND METHODS

Safety Considerations. Ricin is a highly toxic protein that inhibits the cell protein synthesis. Handling requires stringent safety measures. All contact with the substance and with solutions thereof should be avoided, as well as aerosol or dust formation. All preparatory work and handling of pure ricin was performed using dedicated equipment in fume hoods that were located in a separated laboratory with controlled access only for the involved personnel.

Materials. Seeds of different varieties were obtained from Rara växter (Stockholm, Sweden) and from a local garden center (Rijswijk, The Netherlands).

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Table 3. Trypsin Digest Peptides from Chain A of Ricin (GI:132567; Reduced and Carboxymethylated), with Annotation of Sequence Ions According to Roepstorff and Fohlman[35]

T no.[a]	peptide sequence[b]	obsd mass (Da)[c]	obsd sequence ions
T1	IFPK	504.4^{+}	a_2, b_2, $y''-y_3''$, $y_3''-H_2O$
T2	QYPIINFTTAG**ATVQSYTNFIR**	$\mathbf{1225.9^{3+}}$	b_4, $y_1''-y_{11}''$, $[T2+HexNAc+2H]^{2+}$, $[T2+2H]^{2+}$
T3	AVR	$[344.2^{+}]$	
T4	**GR**	$\mathbf{[231.1^{+}]}$	
T5	**LTTGADVR**	$\mathbf{416.7^{2+}}$	a_1, a_2, b_2, $y_1''-y_7''$, $[M+2H-H_2O]^{2+}$, $[M+2H-2H_2O]^{2+}$
T6	HEIPVLPNR	537.8^{2+}	a_2, b_2, b_3, b_3-H_2O, y_1'', $y_3''-y_8''$
T7	**VGLPINQR**	$\mathbf{448.8^{2+}}$	b_2, b_3, $y_1''-y_7''$, $[y_5''+H]^{2+}$, $[y_6''+H]^{2+}$
T8	**FILVELSNHAELSVTLALDVTNAYVVGYR**	$\mathbf{[3205.7^{+}]}$	
T9	**AGNSAYFFHPDNQEDAEAITHLFTDVQNR**	$\mathbf{827.6^{4+}}$	a_2, b_2-b_7, $y_1''-y_{10}''$, $[y_9''+H]^{2+}-[y_{16}''+H]^{2+}$, $[y_{18}''+H]^{2+}$- $[y_{21}''+H]^{2+}$, $[y_{20}''+H]^{3+}-[y_{27}''+H]^{3+}$, $[M+4H-H_2O]^{4+}$
T10	**YTFAFGGNYDR**	$\mathbf{655.8^{2+}}$	a_1, a_2, b_2-b_5, b_3-H_2O, $y_1''-y_{10}''$
T11	**LEQLAGNLR**	$\mathbf{507.3^{2+}}$	a_1, a_2, b_2-b_5, $y_1''-y_8''$, $[y_7''+H]^{2+}$
T12	**ENIELGNGPLEEAISALYYYSTGGTQLPTLAR**	$\mathbf{1147.6^{3+}}$	b_4-b_8, b_{14}-b_{18}, $[b_{27}+H]^{2+}$, $y_1''-y_{15}''$
T13	**SFIICIQMISEAAR**	$\mathbf{820.4^{2+}}$	a_2, b_2-b_9, $y_1''-y_{12}''$
T14	FQYIEGEMR	586.8^{2+}	a_2, b_2-b_5, b_2-NH_3, y_1'', y_2'', $y_4''-y_8''$, $y_8''-NH_3$
T15	TR	$[275.2^{+}]$	
T16	IR	$[287.2^{+}]$	
T17	YNR	$[451.2^{+}]$	
T18	R	$[174.1^{+}]$	
T19	SAPDPSVITLENSWGR	864.9^{2+}	a_2, b_2-b_5, $y_1''-y_{14}''$, $[y_{14}''+H]^{2+}$, PDPSV, PDPSVI, (VI/PD)
T20	LSTAIQESNQGAFASPIQLQR	1130.2^{2+}	b_3-b_7, b_3-H_2O, $y_1''-y_4''$, $y_6''-y_{16}''$
T21	R	$[174.1^{+}]$	
T22	NGSK	$[404.2^{+}]$	
T23	**FSVYDVSILIPIIALMVYR**	$\mathbf{[1116.0^{2+}]}$	
T24	CAPPPSSQF	496.2^{2+}	a_2, b_2-b_8, b_6-NH_3, b_7-NH_3, $y_1''-y_8''$, $[M+H-H_2O]^{+}$, PPPSSQ, PPPSSQF

[a] Trypsin digest peptides numbered from the amino terminal. [b] In boldface type: peptides differentiating ricin from RCA; underlined sequences confirmed by LC−ES MS/MS. [c] In brackets: ions not observed, given as theoretical mass of $[M + H]^{+}$.

All water used in analytical procedures was drawn from a MilliQ system (Millipore, Milford, MA). Acetonitrile (LiChrosolv quality) and formic acid (p.a.) were purchased from Merck (Darmstadt, Germany). Recrystallized α-cyano-4-hydroxycinnamic acid, used as a MALDI matrix, was purchased from Bruker Daltonik (Bremen, Germany). Molecular weight cutoff (MWCO) 10 000 filters were purchased from Amicon (Dronten, The Netherlands). ZipTips were obtained from Millipore. Tris and Tris-HCl (Trizma) were obtained from Sigma (Zwijndrecht, The Netherlands), whereas TPCK-treated trypsin was obtained from Sigma-Aldrich (Steinheim, Germany). Guanidine hydrochloride and EDTA were purchased from Janssen (Geel, Belgium). Sodium 2-iodoacetate and ammonium bicarbonate (99.5+%) were obtained from Fluka Chemie AG (Buchs, Switzerland). Guanidine−Tris−EDTA buffer contained guanidine 6 M, Tris 0.1 M. EDTA 5 mM.

Extraction and Isolation of Ricin. Ricin toxin was purified from castor beans/seeds as earlier described.[23] Briefly, the unshelled and crushed seeds were delipidated by repeated extractions with diethyl ether. After centrifugation at 3000*g*, the pulp was resuspended in distilled water and pH was adjusted to 4.0 by addition of dilute acetic acid. The suspension was homogenized and then centrifuged at 8000*g* for 20 min. The ricin-containing supernatant was extensively dialyzed against distilled water and against 10 mM Tris-HCl (pH 7.7). After centrifugation, ricin was purified by affinity chromatography. The crude ricin extract was loaded onto a galactosyl-Sepharose affinity column. Nonbinding material was eluted by 10 mM Tris-HCl, and ricin was subsequently eluted using a linear gradient from 0 to 0.25 M lactose. The fractions were analyzed by SDS−PAGE, and ricin-containing fractions were pooled.

Further fractionation of ricin toxin was achieved by ion-exchange chromatography on a strong cation-exchange column (UNO S-1, 7 × 35 mm; Bio-Rad) eluted with a linear gradient from 0 to 500 mM NaCl in 10 mM Tris-HCl, pH 7.7. Three fractions containing ricin were isolated. The protein content was determined by absorbance at 280 nm. The fractions were stored at 4 °C until further processing for analysis.

Enzymatic Digestion and Sample Preparation for MS. The sample was enzymatically digested with trypsin, directly on am MWCO filter as described earlier.[24] Approximately 100−200 μg of protein was concentrated on a Microcon centrifugation filter (YM-10, MWCO 10.000; Millipore). The filter was then washed twice by adding 200 μL of 0.2 M ammonium bicarbonate solution and concentrated to a minimal volume. A 300-μL aliquot of a freshly prepared denaturing and reducing solution containing 6 M guanidine hydrochloride, 0.1 M Tris buffer, 5 mM EDTA, and 3 mg/mL DTT or 2 mg/mL BMS was added. The sample was

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Table 4. Trypsin Digest Peptides from Chain B of Ricin (GI:132567; Reduced and Carboxymethylated)

T no.[a]	ricin D peptide sequence[b]	obsd mass (Da)[c]	observed sequence ions
T1	ADVCMDPEPIVR	701.8^{2+}	a_2, b_2-b_6, b_8, b_{10}, b_{11}, $y_1''-y_{10}''$
T2	IVGR	444.3^{+}	a_2, b_2, b_3, $y_1''-y_3''$, $y_2''-NH_3$, $y_3''-NH_3$
T3	**NGLCVDVR**	**467.2^{2+}**	a_2, a_3, b_2, b_3, $y_1''-y_7''$, $[y_5''+H]^{2+}$, $[M+2H-H_2O]^{2+}$
T4	DGR	$[346.2^{+}]$	
T5	**FHNGNAIQLWPCK**	**793.4^{2+}**	a_2, b_2, b_3, b_5-b_{10}, $y_1''-y_8''$, y_{10}'', y_{11}''
T6	**SNTDANQLWTLK**	**695.8^{2+}**	b_2-b_{11}, $(b_{23}-NH_3)-(b_{11}-NH_3)$, $y_1''-y_{10}''$, $[y_{10}''+H]^{2+}$
T7	R	$[174.1^{+}]$	
T8	**DNTIR**	309.7^{2+}	a_1, a_2, a_2-NH_3, b_2, b_3, b_2-NH_3, b_3-NH_3, y_1''-y_4'', y_3-NH_3, y_4''-NH_3
T9	SNGK	$[404.2^{+}]$	
T10	**CLTTYGYSPG VYVMIYDCNTAATDATR**	**1022.4^{3+}**	a_2, b_2-b_8, $b_{10}-b_{15}$, $y_1''-y_{14}''$
T11	WQIWDNGTIINPR	**997.8^{3+}**	a_2, b_2-b_3, b_5, $y_1''-y_7''$, $[T11+HexNAc+2H]^{2+}$, $[T11+2H]^{2+}$
T12	**SSLVLAATSGNSGTTLTVQ TNIYAVSQGWLPTNNTQP FVTTIVGLYGLCLQANSG QVWIEDCSSEK**	**$[7050.8^{+}]$**	
T13	AEQQWALYADGSIRPQQNR	744.4^{3+}	a_2, b_2-b_8, $[y_9''+H]^{2+}-[y_{17}''+H]^{2+}$
T14	**DNCLTSDSNIR**	**648.3^{2+}**	a_1-a_3, b_2-b_5, $y_1''-y_9''$
T15	**ETVVK**	**575.3^{+}**	a_2, b_2-b_4, $y_1''-y_4''$
T16	ILSCGPASSGQR	617.3^{2+}	a_1, a_2, $[a_8+H]^{2+}$, b_2-b_5, y_1'', y_3''-y_{11}'''', $[y_{10}''+2H]^{2+}$, $[y_{11}''+H]^{2+}$
T17	WMFK	306.1^{2+}	a_1, a_2, b_1, b_2, b_1-NH_3, $y_1''-y_3''$, $[M+2H-NH_3]^{2+}$
T18	**NDGTILNLYSGLVLDVR**	**931.5^{2+}**	b_2-b_6, $y_1''-y_{13}''$
T19	**ASDPSLK**	**359.2^{2+}**	a_2, b_2, b_3, b_3-H_2O, $y_1''-y_6''$, $[M+2H-H_2O]^{2+}$
T20	**QIILYPLHGDPNQIWLPLF**	**759.8^{3+}**	$[b_{13}+H]^{2+}-[b_{18}+H]^{2+}$, $y_1''-y_5''$

[a] Trypsin digest peptides numbered from the amino terminal. [b] In boldface type: peptides differentiating ricin from RCA; underlined sequences confirmed by LC−ES MS/MS. [c] In brackets: ions not observed, given as theoretical mass of $[M + H]^{+}$.

incubated at 60 °C for 60 min. A 100-μL sample of a freshly prepared alkylating solution, containing 15 mg/mL iodoacetamide or 70 mg/mL sodium 2-iodoacetate in the 6 M guanidine−Tris−EDTA buffer, was added and the sample was kept at 37 °C for 30 min. The reduced and alkylated sample was spun down and washed twice with 200 μL of 0.2 M ammonium bicarbonate solution. A 300-μL sample of the digestion buffer was added containing trypsin at an enzyme-to-substrate ratio of ~1:50, and the sample was digested at 37 °C overnight. The peptides were recovered by spinning down the digest solution to a minimal volume and twice washing the filter with 50 μL of a 1:1 mixture of 0.2 M ammonium bicarbonate solution and acetonitrile. Before analysis by LC−ES MS, 5 μL of formic acid was added to the sample.

MALDI MS. All MALDI MS experiments were conducted on a Biflex III reflectron time-of-flight instrument (Bruker, Bremen, Germany), equipped with delayed extraction and with a UV ionization laser (N_2, 337 nm). To an aliquot of a crude bean extract or its trypsin digest, an equal volume of 5% aqueous formic acid was added. Of that mixture, 10 μL was flushed a few times over a single ZipTip; the column material of that tip was then flushed with 10 μL of a 0.1% TFA solution and the peptides or proteins were eluted with 10 μL of matrix solution (saturated α-cyanohydroxycinnamic acid with 0.1% TFA in acetonitrile/water, 1:2 v/v). Of the matrix−sample mixture, 0.5 μL was brought on target.

HPLC−ES MS/MS. LC−ES MS(/MS) experiments were conducted on a Q-TOF hybrid instrument (Micromass, Altrincham, U.K.) equipped with a standard Z-spray ES interface (Micromass) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA), or on an Micromass Autospec OA-TOF (Micromass). The chromatographic hardware for both systems consisted of a precolumn splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a six-port valve (Valco, Schenkon, Switzerland) with a 10- or a 50-μL injection loop mounted and a PepMap C_{18} column (15 cm × 300 μm i.d., 3-μm particles; LC Packings).

A gradient of eluents A (H_2O with 0.2 vol % formic acid) and B (acetonitrile with 0.2 vol % formic acid) was used to achieve separation as follows: 100% A (at time 0 min, 0.1 mL/min flow) to 100% A (at 5 min, 0.5 mL/min flow) to 20% A and 80% B (at 60 min, 0.5 mL/min flow). The flow delivered by the liquid chromatograph was split precolumn to allow a flow of ~6 μL/min through the column and into the ES MS interface.

The Q-TOF was operated at a cone voltage of 25−35 V, employing nitrogen as the nebulizer and desolvation gas (at a flow of 20 and 400 L/h, respectively). MS/MS product ion spectra were recorded using a collision energy between 14 and 30 V, with argon as the collision gas (at an indicated pressure of 10−4 mbar). LC−ES MS(/MS) was used for trypsin and pepsin digest analyses.

RESULTS AND DISCUSSION

Mass Spectrometric Screening of Crude Toxin. A crude aqueous extract of a single *R. communis* bean of unknown variety was prepared. The crude extract was subjected to reduction, cysteine derivatization, and trypsin digestion. The digested material was then subjected to MALDI MS analysis. The MALDI spectrum of crude, intact ricin (shown in Figure 1) is sufficiently informative to allow screening. The $[M + H]^{+}$ signal is wide, due

Table 5. Peak Attribution of Peaks in MALDI Spectrum from S-Carboxymethylated Trypsin-Digested Crude Ricin

observed Da (relative intensity, %)	calcd Da	attribution(s) [a]
956.5 (2)	956.5	T4AS
1013.6 (1)	1013.6	T11A
1042.5 (1)	1042.6	T10−11AP
1074.6 (5)	1074.6	T6A, T5RA
1135.5 (5)	1135.5	T17AP
1172.6 (12)	1172.5	T14A, T12RA
1213.6 (10)	1213.6	T2AS
1233.6 (5)	1233.6	T16B, T19RB
1295.6 (1)	1295.6	T14B
1310.6 (4)	1310.6	T10A
1402.6 (4)	1402.6	T1B, T1RB
1533.7 (2)	1533.8	T4RB
1639.8 (10)	1639.8	T13A
1643.7 (9)	1643.8	T11RA
1728.8 (37)	1728.9	T19A, T17RA
1852.7 (2)	1852.8	T7AL
1861.9 (3)	1862.0	T18B
1888.9 (16)	1889.0	T21RB
1951.8 (2)	1952.1	T6−7A
1999.7 (8)	1999.9	T7−8AL
2230.9 (16)	2231.1	T13B, T16RB
2259.0 (47)	2259.2	T20A, T18RA
2305.0 (23)	2305.1	T10−11A
2535.1 (100)	2535.2	T6AL
2797.1 (4)	2797.3	T11−12RA, T3RB
2991.2 (6)	2990.6	T1−2A[b]
3065.2 (2)	3065.3	T10B
3153.3 (1)	3152.7	T17−19B
3307.4 (3)	3307.5	T9A
3366.3 (2)	3365.5	T14−15AP

[a] Trypsin digest peptides numbered from the amino terminal of the A-chain (suffix A), B-chain (B), RCA A-chain (RA), RCA B-chain (RB), 2S albumin propeptide (AP), small chain (AS), and large chain (AL). [b] Note that T2A would be glycosylated and that the match to T1−2A is probably a coincidence.

to both a relatively poor mass resolution in linear TOF and a wide distribution of molecular masses due to biovariation, even within a single bean.

This kind of screening gives coarse information on the sample, and more detailed analysis is required to obtain irrefutable proof of the presence of ricin. The access to irrefutable proof was first studied with purified ricin and later with crude ricin preparations.

Mass Spectrometry of Purified Ricin. The molecular structure of ricin differs at several levels: distinction between ricin D and ricin E, in the biovariation in the ricin amino acid backbone ("isoforms"), and in the glycosylation pattern ("glycoforms"). These distinctions are important for accurate identification of the toxin.

The distinction between ricins D and E is based on hybrids of ricin and a protein known as *R. communis* agglutinin (RCA). Like a molecule of ricin, a single molecule of RCA is formed by an A-chain and a B-chain, linked by a disulfide bridge. RCA is highly similar to ricin. The sequence homology of the A-chains is 94%, whereas that of the B-chains is 85%.[25] Both ricin and RCA are biosynthesized as single molecules, known as preproricin and prepro-RCA, respectively. Posttranslational proteolytic processing of these single molecules leads to the mature, two-chain proteins. On the basis of sequence evidence, it was proposed that natural genetic recombination has led to the occurrence of hybrid precursor proteins, with ricin A-chain and a mixed ricin/RCA B-chain.[25] The hybrid is known as ricin E, whereas the nonhybrid is known as ricin D. Some of the *R. communis* plants, notably those producing large seeds, produce only ricin D, whereas other plants produce both ricins D and E.[26] Since the B-chain is responsible for cell adherence and internalization of the toxin molecule, ricin D and ricin E differ slightly in their activity through a difference in affinity for certain cells,[27] whereas the overall toxicity is similar.[28]

Conflicting data on the amino acid backbone of ricin, other than the ricin D/E distinction, are also reported. In 1978 and 1979, the first complete amino acid sequences of ricin A-chain and B-chain were reported.[29,30] There is some sequence difference with later, gene or X-ray diffraction-derived amino acid sequences of ricin A- and B-chain; in the present study, the gene-derived amino acid sequences are used for position reference (see also Tables 1 and 2).

Ricin glycoforms come about from posttranslational modification of preproricin. In the modification process, preproricin has to lose the N-terminal signal (35 amino acid residues) and a 12-residue peptide between the future A-chain C-terminal and future B-chain N-terminal, by proteolytic cleavage (see, for example, ref 31). Prior to proteolysis, the protein backbone is N-glycosylated, by enzymatic addition of sugar molecules to specific asparagine residues. As far as it is now known, the ricin residues that bare N-glycans are invariable (N10, sometimes N236 of the A-chain,[32] and N95 and N135 of the B-chain[33]), but the glycan compositions themselves are variant.

Ricin was extracted from castor beans obtained from different varieties of *R. communis* plants. The affinity-purified material was fractionated on a strong cation-exchange column eluted with a sodium chloride gradient (Figure 1). Three ricin-containing fractions were obtained from most of the plant varieties, except for *R. communis zanzibariensis,* which gave only one ricin peak. This is consistent with earlier observations on the occurrence of ricin variants, where large grain varieties typically contain the D form and small grain varieties the E and D forms. The differences in amino acid sequence and the heterogeneity of the glycosylation do result in some separation of the glycoforms during ion-exchange chromatography (Figure 2).

The ricin-containing fractions were digested with trypsin, after reduction and alkylation of the cysteine residues. The digests were analyzed by LC−ES MS and MS/MS, and the product ion spectra were evaluated and compared with the sequence ions expected for ricin trypsin digest peptides from database amino acid

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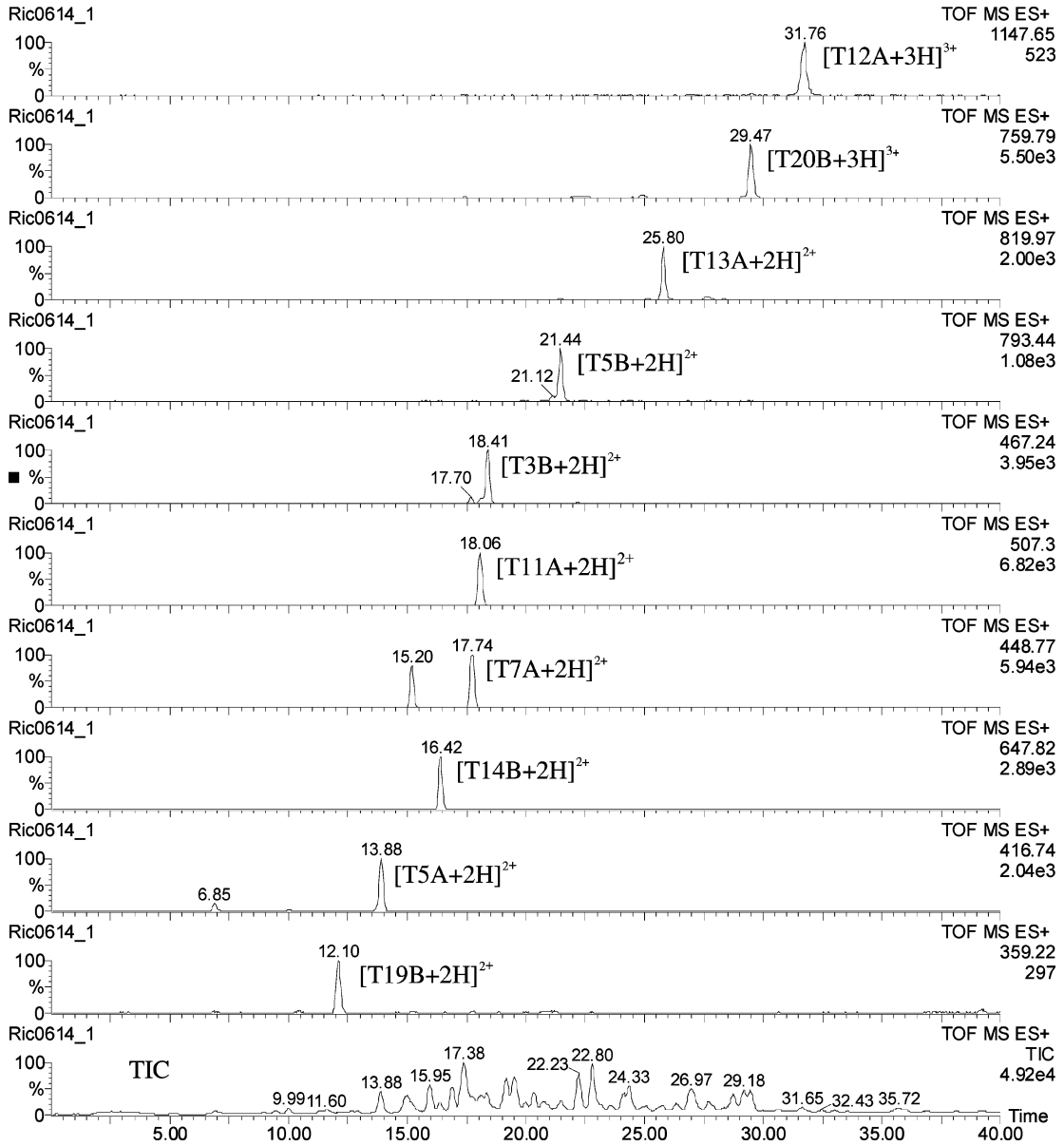

Figure 3. LC−ES MS of a reduced, carbamidomethylated, and trypsin-digested crude ricin extract from a *R communis impala* seed. Total ion chromatogram and extracted ion chromatograms of ricin-specific peptides.

sequences. Results obtained with the Autospec and the Q-TOF instruments were nearly identical; all data in this report are from Q-TOF experiments.

A-Chain. Table 1 gives a comparison of the A-chain amino acid sequences of ricin[31] and RCA[34] found in Swissprot with the sequence obtained already in 1978 (GI:223026).[29] The early ricin sequence differs from the Swissprot sequence at five positions, of which two are most probably due to flaws in the protein sequencing methods used at that time (R235, neighbor to R234, two adjacent trypsin cleavage sites; L254, neighbor to M255, a cyanogen bromide cleavage site). The I249 residue cannot be differentiated from the L reported in the alternative sequence by the MS/MS methods used here.

The sequence ions obtained for the A-chain peptides are listed in Table 3, following common nomenclature.[35] The peptides were found in all fractions 1, 2, and 3 from the ricin varieties investigated and agree with database data for chain A of ricin (GI:132567, GI: 476541, GI:21083).

B-Chain. Differences in the B-chain amino acid sequence of the ricin D and E isoforms are localized in the C-terminal half (see Table 2). In addition, the C-terminal half of ricin E B-chain is identical to that of RCA. For the 1978 protein sequencing data of ricin D, conflicting sequences have been attributed to the loss of tryptophan in sample preparation for on Edman sequencing.[36] Other differences are less easily attributed to artifacts. Present MS/MS data for T10 (Table 4) confirms the sequence S70P71 (NCBI entries GI:132567, GI:251804, GI:476541, and GI:21083) and counters P70S71 (entry GI:224694). The present data also clearly indicate A237 (see T19 in Table 4, GI: 132567) instead of R237 (GI:251804).

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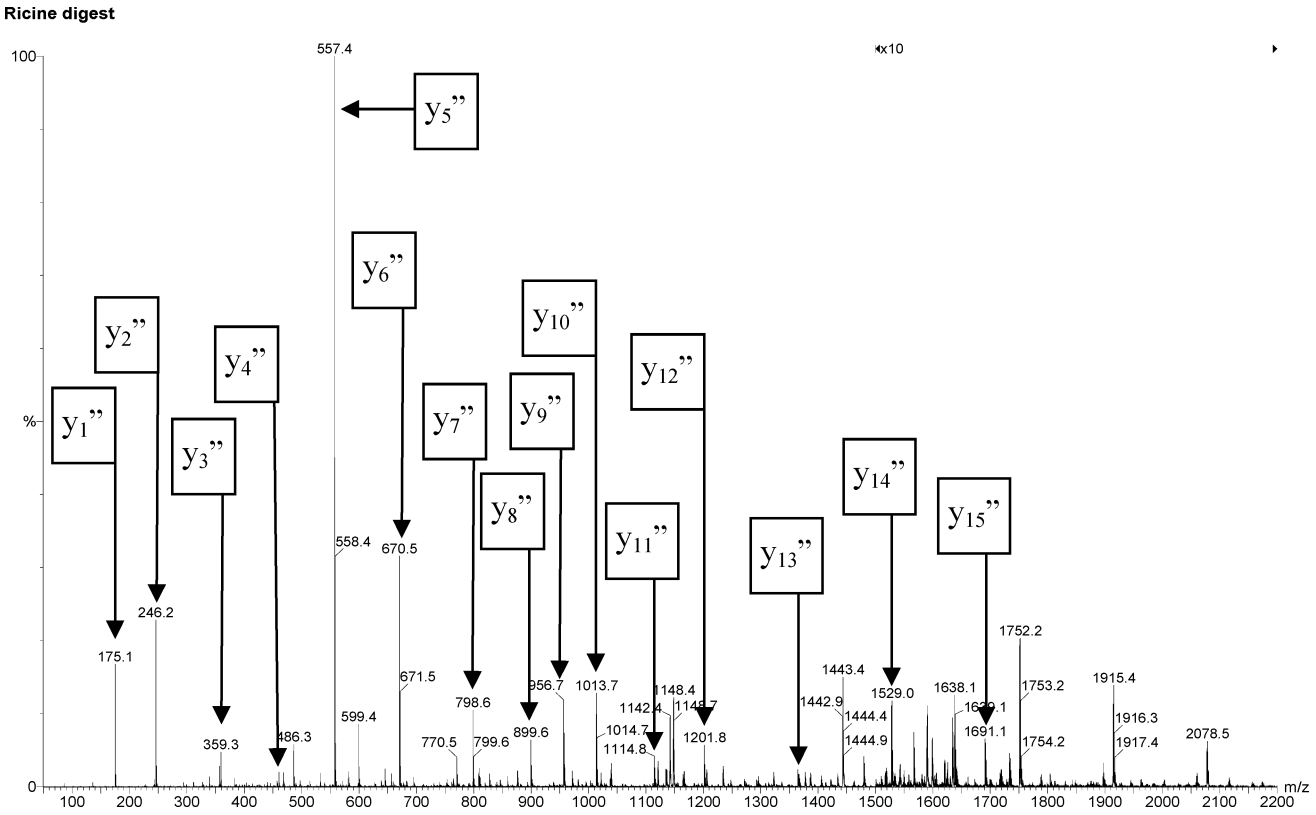

Figure 4. LC−ES MS/MS product ion spectrum of precursor ions 1147.6^{3+}, identified by the sequence ions as [T12A + 3H]$^{3+}$ with sequence ENIELGNGPLEEAISAL*YYYSTGGTQLPTLAR*. For clarity, only y″series ions are labeled.

With the observations made for ricin A- and B-chains, the fractions from ion-exchange chromatography (Figure 2) were analyzed by LC−ES MS/MS. The peptide sequences obtained showed that fraction 1 contained ricin D and that fractions 2 and 3 both contained ricin E.

*Choice of Ricin Marker Peptides***.** Unambiguous identification of ricin requires evidence for the presence of the two chains and distinction from RCA and any other proteins. From Tables 3 and 4 it is seen that the A-chain of ricin is selectively covered by its T5, T7−T13, T23, and glycosylated T2, whereas the B-chain is selectively covered by its T3, T5, T6, T10, T14, T18−T20, and glycosylated T11 and T12. It is noted that the active site containing digest peptide T14A equally occurs in ricin D and E and RCA. These distinctive peptides are all more than seven amino acid residues long, and most are unique to ricin when matched against the NCBInr database; A-chain T5 also appears in a *Pomacea flagellata* hemagglutinin (GI:46395685),[37] whereas B-chain T3 also appears in a family of type 2 ribosome-inactivating proteins in *Cinnamomum camphora* (GI:15081584, 15081586, 15081588).[38] Of all the ricin-specific peptides, the B-chain T12, T14, T18, T19, and T20 are specific to ricin D.

T5A, T7A, T10A−T13A, T23A, T3B, T5B, T6B, T14B, and T18B−T20B were observed in the experiments with ricin purified from different varieties and with crude ricin preparations. T12B was not detected. Sequence ions were obtained for T11B, but they cover only part of the sequence.

Mass Spectrometry of Crude Toxin. The MALDI mass spectrum of trypsin-digested ricin (not shown) is summarized in Table 5. A ProFound[39] search with the initial spectrum matched ricin, and a subsequent search with a spectrum minus the assigned ricin signals matched the *R. communis* 2S albumin (GI:112762). Remaining signals of RCA were then assigned by hand. These search results show that MALDI MS analysis of a trypsin digest of crude ricin produces sufficient information for a quick screening in addition to analysis of the intact toxin.

LC−ES MS/MS peptide sequencing is more powerful than peptide mapping, when protein mixture analysis is concerned. Based on the results obtained with purified ricin samples, a number of peptides were chosen as ricin markers for selective screening of ricin in the crude extract. A "data-dependent" LC−ES MS/MS experiment was set up to search for selected precursors and acquire product ion spectra of 10 ricin-specific peptides in crude trypsin digests. Five ionized peptides from each of the A and B chain were selected: T5A, T7A, T11A−T13A, T3B, T5B, T14B, T19B, and T20B. Figure 3 gives typical chromatograms from a trypsin-digested crude extract of an *R. communis impala* bean. For the experiment, an estimated amount of 10 pmol of ricin was injected onto the column. Survey scans were acquired over the mass range 400−1500 in 0.5 s, and mass and peak presence for the preprogrammed mass and charge state were evaluated by the software. When a mass within the accuracy window of ±0.1 Da was detected above the intensity threshold level, a 5-s acquisition of the product ion spectrum was triggered

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Table 6. Summary of LC–ES MS/MS Derived Sequence Information. Annotation of Characteristic Ions According to Roepstorf–Fohlman Nomenclature[35]

digest peptide[a,b]	sequence[c]	obsd mass (Da)	observed fragment ions
ricin[d]			
T1 A	IFPK	504.4^{+}	a_2, b_2, $y_1''-y_3''$, $y_3''-H_2O$
T5 A	LTTGADVR	416.7^{2+}	a_1, a_2, b_2, $y_1''-y_7''$, $[M+2H-H_2O]^{2+}$, $[M+2H-2H_2O]b^{2+}$
/T5 A	TGADVR	309.7^{2+}	a_1, a_2, b_2-b_4, $y_1''-y_5''$
T6 A	HEIPVLPNR	537.8^{2+}	a_2, b_2, b_3, b_3-H_2O, y_1'', $y_3''-y_8''$
T7 A	VGLPINQR	448.8^{2+}	b_2, b_3, $y_1''-y_7''$, $[y_5''+H]^{2+}$, $[y_6''+H]^{2+}$
T9 A	AGNSAYFFHPDNQEDAEAITHLFTDVQNR	1103.2^{3+}	b_{25}, $y_4''-y_{11}''$, $[y_{20}''+H]^{2+}-[y_{23}''+H]^{2+}$
T10 A	YTFAFGGNYDR	655.8^{2+}	a_1, a_2, b_2-b_5, b_3-H_2O, $y_1''-y_{10}''$
T11 A	LEQLAGNLR	507.3^{2+}	a_1, a_2, b_2-b_5, $y_1''-y_8''$, $[y_7''+H]^{2+}$
T12 A	ENIELGNGPLEEAISALYYYSTGGTQLPTLAR	1147.6^{3+}	b_4-b_8, $b_{14}-b_{18}$, $[b_{27}+H]^{2+}$, $y_1''-y_{15}''$
T13 A	SFIIZIQMISEAAR	820.4^{2+}	a_2, b_2-b_9, $y_1''-y_{12}''$
T14 A	FQYIEGEMR	586.8^{2+}	a_2, b_2-b_5, b_2-NH_3, y_1'', y_2'', $y_4''-y_8''$, $y_8''-NH_3$
T14 A[Mx]	FQYIEGEM(−$HSCH_3$)R	562.8^{2+}	a_1, a_2, b_2-b_5, $y_3''-y_8''$
T19 A	SAPDPSVITLENSWGR	864.9^{2+}	a_2, b_2-b_5, $y_1''-y_{14}''$, $[y_{14}''+H]^{2+}$, PDPSV, PDPSVI, (VI/PD)
T20 A	LSTAIQESNQGAFASPIQLQR	1130.2^{2+}	b_3-b_7, b_3-H_2O, $y_1''-y_4''$, $y_6''-y_{16}''$
T24 A	ZAPPPSSQF	496.2^{2+}	a_2, b_2-b_8, b_6-NH_3, b_7-NH_3, $y_1''-y_8''$, $[M+H-H_2O]^{+}$, PPPSSQ, PPPSSQF
T1 B	ADVZMDPEPIVR	701.8^{2+}	a_2, b_2-b_6, b_8, b_{10}, b_{11}, $y_1''-y_{10}''$
/T1B	DVZMDPEPIVR	666.3^{2+}	a_2, b_2-b_5, y_1'', y_4'', $y_6''-y_9''$
T3 B	NGLZVDVR	467.2^{2+}	a_2, a_3, b_2, b_3, $y_1''-y_7''$, $[y_5''+H]^{2+}$, $[M+2H-H_2O]^{2+}$
T5 B	FHNGNAIQLWPZK	793.4^{2+}	a_2, b_2, b_3, b_5-b_{10}, $y_1''-y_8''$, y_{10}'', y_{11}''
T6 B	SNTDANQLWTLK	695.8^{2+}	b_2-b_{11}, $(b_{2,3}-NH_3)-(b_{11}-NH_3)$, $y_1''-y_{10}''$, $[y_{10}''+H]^{2+}$
T8 B	DNTIR	309.7^{2+}	a_1, a_2, a_2-NH_3, b_2, b_3, b_2-NH_3, b_3-NH_3, $y_1''-y_4''$, y_3-NH_3, $y_4''-NH_3$
T10 B	ZLTTYGYSPGVYVMIYDZNTAATDATR	1022.4^{3+}	a_2, b_2-b_8, $b_{10}--b_{15}$, $y_1''-y_{14}''$
T13 B	AEQQWALYADGSIRPQQNR	744.4^{3+}	a_2, b_2-b_8, $[y_9''+H]^{2+}-[y_{17}''+H]^{2+}$
T14 B	DNZLTSDSNIR	648.3^{2+}	a_1-a_3, b_2-b_5, $y_1''-y_9''$
T16 B	ILSZGPASSGQR	617.3^{2+}	a_1, a_2, $[a_8+H]^{2+}$, b_2-b_5, y_1'', $y_3''-y_{11}''$, $[y_{10}''+2H]^{2+}$, $[y_{11}''+H]^{2+}$
T17 B	WMFK	306.1^{2+}	a_1, a_2, b_1, b_2, b_1-NH_3, $y_1''-y_3''$, $[M+2H-NH_3]^{2+}$
T18 B	NDGTILNLYSGLVLDVR	931.5^{2+}	b_2-b_6, $y_1''-y_{13}''$
T19 B	ASDPSLK	359.2^{2+}	a_2, b_2, b_3, b_3-H_2O, $y_1''-y_6''$, $[M+2H-H_2O]^{2+}$
T20 B	QIILYPLHGDPNQIWLPLF	759.8^{3+}	$[b_{13}+H]^{2+}-[b_{18}+H]^{2+}$, $y_1''-y_5''$
T20 B	QIILYPLHGDPNQIWLPLF	1139.1^{2+}	$b_{14}-b_{16}$, $[b_{14}+H]^{2+}-[b_{18}+H]^{2+}$, $y_2''-y_5''$,
RCA[d]			
T6 A	VGLPISQR	435.3^{2+}	a_3, a_3-NH_3, b_2-b_4, $y_1''-y_7''$
T10 A	ENIELGTGPLEDAISALYYYSTZGTQIPTLAR	1173.7^{3+}	b_3-b_7, $[b_{26}+H]^{2+}$, $[b_{27}+H]^{2+}$, $y_1''-y_{12}''$
T3 B	NGLZVDVTGEEFFDGNPIQLWPZK	933.2^{3+}	b_3-b_8, $[b_{16}+H]^{2+}-[b_{21}+H]^{2+}$, $y_1''-y_{12}''$, $[y_{19}''+H]^{2+}-[y_{21}''+H]^{2+}$, PIQ
T5–6 B	KDSTIR	360.2^{2+}	y_1'', $y_3''-y_5''$
T11 B	TIINPTSGLVLAATSGNSGTK	1001.6^{2+}	a_2, b_2-b_{12}, y_1'', $y_3''-y_{18}''$
T13 B	VWLEDZTSEK	634.3^{2+}	a_1, a_2, b_2-b_5, $y_1''-y_9''$, $[y_8''+H]^{2+}$, $[y_9''+H]^{2+}$
Y13/*T*13 B	LEDZTSEK	481.2^{2+}	a_1, a_2, b_2, b_3, $y_1''-y_7''$
T15 B	DNZLTTDANIK	633.2	a_2, b_2-b_{10}, $y_1''-y_9''$
Y18/*T*19 B	NGLVLDVR	443.3^{2+}	a_2, b_2-b_5, $y_1''-y_7''$
T20–21 B	RSDPSLK	401.7^{2+}	a_1, a_5, a_6, b_3-b_6, $y_1''-y_4''$
/T22	HGNLNQIWLPLF	726.4^{2+}	b_6-b_9, $[b_9+H]^{2+}$, $[b_{10}+H]^{2+}$, $y_1''-y_5''$, PL/LP
T22	QIIVHPVHGNLNQIWLPLF	746.8^{3+}	$[b_{12}+H]^{2+}-[b_{18}+H]^{2+}$, $y_1''-y_5''$
2S Albumin[d]			
T4 P	EGSSSQQZR	520.2^{2+}	a_1, b_2, b_3, $y_1''-y_8''$, $y_4''-NH_3$
T5 P	QEVQR	330.2^{2+}	a_1, a_2, b_2, b_3, b_2-NH_3, b_3-NH_3, $y_1''-y_4''$, $y_2''-NH_3$, $y_3''-NH_3$, $[M+H-NH_3]^{+}$
T7 P	DLSSZER	434.2^{2+}	a_2, b_2-b_4, $y_1''-y_5''$, $[y_5''+H]^{2+}$, $[y_6''+H]^{2+}$
T17 P	AGEIVSSZGVR	568.2^{2+}	a_2, b_2-b_6, y_1'', $y_3''-y_8''$
T1 S	PSSQQGZR	417.2^{2+}	a_2, b_2, y_1'', $y_3''-y_6''$
T2 S	GQIQEQQNLR	607.3^{2+}	b_2-b_9, y_1''-y_8'', $[y_7''+H]^{2+}$, $[y_8''+H]^{2+}$
T3 S	QZQEYIK	485.2^{2+}	$(b_2-NH_3)-(b_6-NH_3)$, y_1''-y_6'', y_5''-NH_3
T4 S	QQVSGQGPR	478.7^{2+}	b_2, b_3, $(b_2-NH_3)-(b_7-NH_3)$, y_1''-y_7''
T3 L	GZZDHLK	446.2^{2+}	a_2, b_2, b_5, b_6, y_1''-y_5'', $[y_5''+H]^{2+}$, $[y_6''+H]^{2+}$, ZDHL
T7–8 L	TAANLPSMZGVSPTEZRF	1001.1^{2+}	a_2, b_2-b_5, b_{11}, b_{12}, y_1''-y_{15}'', $[y_{13}''+H]^{2+}$, $[y_{14}''+H]^{2+}$

[a] In reference to separate A- and B-chains for ricin and RCA, in reference to chains for 2S albumin (P for propeptide, S for small chain, L for large chain). [b] Trypsin digest fragments, T, numbered from N-terminal, with Y for chymotrypsin cleavage, / for anomalous termination, and Mx for deviant methionine. [c] In reference to ricin sequence from NCBInr 2AAI (GI:494726), RCA A-chain sequence from RLCSAG (GI:476540), RCA B-chain sequence from 1209171A (GI:225114), and 2S albumin from P01089 (GI:112762). [d] Ricin common to ricin and RCA, RCA exclusive to RCA and albumin exclusive to 2S albumin.

before switching back to the survey scan. Normally 7−10 of the marker peptides were detected in the crude trypsin digests. Reasons for the failure to acquire a product ion spectrum were (1) too low intensity of the marker peptide or (2) coelution with other peptides in the crude digest.

For a full characterization of the sample, the trypsin-digested crude extract was analyzed repetitively and the product ion spectra of most of the intense peptides were acquired. Figure 4 gives a typical spectrum, whereas Table 6 summarizes the results from LC−ES MS/MS experiments. In that table, all peptides that are common to ricin and RCA are given under "ricin". It is noted that peptides from a third protein, *R. communis* 2S albumin, were found along with those of RCA and ricin.

These results show that ricin can irrefutably be identified, even in the presence of RCA or *R. communis* albumin. RCA and the albumin provide circumstantial evidence of the identity of ricin, in an unequivocal reference to the source of the material. The sequence coverage of ricin is over 70%, not considering the information that can be gained from glycopeptides.

Although we have shown that MALDI MS screening and subsequent LC−ES MS/MS identification may be used in forensic determination of ricin, it may be argued that the presence of functional ricin must be proven. Proof of toxic activity can be obtained from an in vitro activity test such as the protein synthesis-inhibiting test, with nuclease-treated rabbit reticulocyte lysate and luciferase mRNA,[40] or a cell toxicity test that verifies both cell entry and protein-inhibiting activity.[41]

CONCLUSIONS

It was shown that ricin can unequivocally be identified by LC−ES MS/MS experiments with reduced, cysteine-derivatized, trypsin-digested material. It was also shown that MALDI MS can be used to prove the presence of intact ricin and to screen samples for ricin peptides. The amount of crude sample material required was a few milligrams with less than 5% ricin. To improve the sensitivity and the efficiency of the method, a selection of a few marker peptides from the A and B chains can be used to establish an LC−MS/MS screening method. A suitable choice of ricin-specific trypsin digest peptides is T5, T7, T11, T12, and T13 from the A-chain and T3, T5, T14, T19, and T20 from the B-chain. This would allow a single analysis run per sample at maximum instrument detection sensitivity.

ACKNOWLEDGMENT

Skillful technical assistance by Crister Åstot and Yvonne Nygren are gratefully acknowledged. B.L.M.v.B., A.L.d.J., and A.G.H. thank The Netherlands Ministry of Defence for continuous support. S.-Å.F. and C.N. thank the Swedish Ministry of Defence for financial support.

Received for review August 20, 2004. Accepted November 4, 2004.

AC048756U

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