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CHARACTERIZATION OF NATIVE AND DENATURED RICIN USING MALDI-TOF/MS

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Abstract – Ricin is a toxic protein present in the seeds of castor bean plant. It can be inactivated by heat; therefore characterization of denatured ricin is essential to differentiate it from native ricin and to avoid any ambiguity in its identification. In this study, potential of mass spectrometry using MALDI-TOF/MS has been exploited to investigate the effects of heat treatment on ricin and spiked food matrices. The molecular weights of ricin, ricin A (A_1 and A_2) and B chain were found to be 62.8 kDa, 31.2 kDa, 32.5 kDa and 32 kDa respectively. The mass spectrum revealed a polypeptide chain of 11.1 kDa for denatured ricin. The peptide mass fingerprinting showed 24 peptides, six were common both in native and denatured ricin. The differentiating peptide at position 294-318 (*m/z* 934.533) was observed only in denatured ricin. The three selected marker peptides m/z 1013.6, 1310.7, 1728.9 are chosen for identification of ricin inactivated by heat in spiked apple juice and milk samples by immunocapture analysis. There is always a probability of denatured non- toxic ricin being confused with native (toxic) ricin to create unnecessary panic. Keeping this probability in mind, our study will be of immense value in minimising such risk.

Key words: Ricin, denatured, ambiguity, MALDI-TOF/MS, peptide mass fingerprinting, marker, immunocapture

INTRODUCTION

Ricin toxin (RT) is a highly potent, ribosome-inactivating toxin present in the castor bean. It is a glycoprotein lectin and possesses a heterodimeric structure with a molecular weight of approximately 65 kDa. It comprises of a catalytically active A-chain (or RTA, 29-32kDa) disulfide bonded to a galactose binding lectin B chain (or RTB, 32-34 kDa) responsible for cellular binding and internalization of the toxin (31). Ricin A chain exhibits RNA N-glycosidase activity that hydrolyses a specific adenine residue present at 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 the complete inhibition of the cellular translation and cell death (7).

Ricin is highly toxic, with the estimated lethal dose (LD_{50}) to animals and humans in the μg/kg range (33). Toxicity mainly results from the inhibition of protein synthesis, but other reported mechanisms include disturbance in calcium and magnesium balance, (30).

It is known that ricin can exist in the same plant, in different isoforms depending on the type of beans and plant variety from which the same is purified. Ricin may vary in degree of glycosylation (18) between the different plant species of castor bean as well as within the same plant because of multigenic expression. There are several isoforms of ricin, including ricin D, ricin E and the closely related *Ricinus communis agglutinin* (RCA), encoded by a small multigene family of approximately eight numbers, some of which are non-functional (35). Funatsu and his coworkers (25) isolated two ricin variants, named as ricin D and ricin E differing in their amino

Abbreviations: **kDa**, kilodalton; **LD50**, lethal dose 50; **RCA**, Ricinus communis agglutinin ; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis; **DTT**, Dithiothreitol; **TEMED**, Tetramethylethylenediamine; **2-DE**, Two-dimensional gel electrophoresis, **PMF**, ***probability mass function***

acid sequence, isoelectric points, affinity to Sepharose 4B and cytotoxicity towards cultured cells. RCA is a tetramer, each molecule consists of two ricin equivalents held together by non-covalent forces. RCA is not directly cytotoxic but does have affinity for the red blood cells, leading to agglutination and subsequent haemolysis (1).

The relatively easy production of ricin by extraction from the bean, its wide availability, and the lack of specific treatment of ricin poisoning make it a potential bioweapon for terrorist use. Thus, the development of rapid and specific ricin detection methods is of absolute necessity. The characterization of ricin and its subunits has been attempted in many ways. SDS-PAGE is conventionally used for assessing purity and subunit composition of proteins but often do not provide correct results because commercial dyes used for visualizing proteins do not bind to all proteins in a stoichiometric manner (37). Moreover it gives insufficient resolution for glycosylated proteins. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) is a recently developed mass spectrometric technique providing an accurate, powerful characterization and identification of peptides and proteins (17).

Mass spectrometry is one of the advanced techniques that have been used to analyze ricin and investigate component proteins and peptides from the castor bean extracts either by MALDI-TOF/MS or LC-ESI/MS (3, 26, 32). These different methodologies are cantered on the identification or characterization of ricin. The identification of tryptic fragments of ricin was first reported by van Barr and co-workers (8), who thoroughly demonstrated that ricin could be specifically identified through mass spectrometric analysis by its tryptic digestion. Recently Kalb and Barr (16) had described mass spectrometric detection of ricin and its activity in food and clinical samples. Duriez et al. (5) developed an MS-based analytical method using immunocapture for specific and sensitive detection of ricin in the complex environmental samples. The use of electrospray mass spectrometry (ESI/MS), capillary zone electrophoresis (CE) and resonant mirror to analyze, characterize and differentiate between different varieties of ricin toxins has been reported (4).

Characterization of native ricin has been demonstrated by MALDI-TOF/MS but there are no such reports for heat denatured ricin (3, 38). Thermal treatments are known to cause ricin denaturation with loss of tertiary structure (23). Several reports indicate that ricin can be detoxified by thermal treatment (15, 29); however, the conditions required for inactivation are not well defined. We had earlier reported that toxicity of native ricin is lost as it is heated at 100°C for different time intervals. Also, all the immunological methods are able to identify native and denatured ricin but could not distinguish them (20). Analytical techniques like MALDI-TOF/MS, LC-ESI/ MS and MS-MS can recognize ricin specific peptides and may distinguish native/ denatured ricin.

Limited information exists on the stability and detection of ricin added to foods before and after processing by immunological methods and real time –PCR (12, 34). We present here the results on the thermal stability and detection of ricin when present alone or in food samples by MALDI-TOF/MS. The aim of the present study was to (1) characterize native and denatured ricin; (2) distinguish between native and denatured ricin and (3) detect denatured ricin in food samples by MALDI-TOF/MS.

MATERIALS AND METHODS

Chemicals

Bio-gel (A-0.5m gel) was obtained from the Bio-Rad, USA, CM Sepharose Fast Flow, molecular weight markers, trypsin, 3, 5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4-hydroxycinnamic acid (CHCA), acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Sigma Chemicals Co. (St. Louis, MO). The castor seeds were purchased from the local market. Milk and apple juice were purchased from a local grocery store. IPG strips (Bio-Rad, USA) for isoelectric focusing. Other reagents and buffers were procured from Bio-Rad laboratories, USA and SRL/Himedia/Merck (India).

Purification of ricin

Ricin toxin was extracted and purified from the seeds of *Ricinus communis* as earlier described (21). Decorticated seeds were crushed and delipidated by repeated extractions with ether. After centrifugation at 4000g for 10 min, the pulp was soaked in 5% acetic acid and left overnight. The suspension was homogenized and then centrifuged at 8000g for 10 min. Supernatant was collected, adjusted to 60% ammonium sulfate saturation, left overnight at 4°C and centrifuged at 10,000g for 30 min. Pellet was collected and dissolved in distilled water and dialyzed against distilled water. The dissolved pellet was concentrated by lyophilization and the resulting crude ricin extract was subjected to gel filtration chromatography for separation of lectins based on their molecular size. Chromatographic purification was achieved using protein purification system, Bio-Rad, USA (Biologic-HR model). The crude ricin was loaded onto a column (120 x 1.73 cm) packed with bio-gel A -0.5m gel. The column was washed with 0.05 M PBS, pH-7.2, containing 0.1 M NaCl and the matrix-bound lectins were eluted with the same buffer. Protein containing fractions were pooled and concentrated.

Purification of A and B subunits

Separation of A and B subunits of ricin was done according to the method of Emmaneul et al. (6). Protein (14.8 mg/1.25ml) in 0.05 M Tris/HCl, pH 8.5, was incubated with 0.05 M DTT under nitrogen at 37°C for 3 hr. After cooling to 4°C the reduced proteins were loaded onto lactamyl sepharose column packed as described by Hegde and Podder (14). Biologic-HR was used for chromatographic separation of ricin A and B chain. Fractions were collected and absorbance was recorded at 280 nm. The A chain fraction eluted with 0.05 M Tris, pH 8.5, 0.5 M $MgCl_2$, 0.01 M DTT, and the B chain fractions with the same buffer supplemented with 0.4 M lactose. The A and B fractions were pooled separately, dialyzed against distilled water and lyophilized.

Denaturation of ricin

Ricin was denatured in boiling water bath at 100°C for 3.75, 7.5, 15, 30, and 60 min. After boiling for particular time period samples were cooled at room temperature and subjected to further studies.

One-dimensional electrophoresis

SDS-PAGE was performed under reduced and non-reduced conditions to assess the purity of ricin, and its subunits using Bio-RAD, USA electrophoretic apparatus. The native and denatured ricin samples were also studied on SDS- PAGE gel. The SDS-PAGE was performed according to Laemmli (22).

Acidic non-denaturing gel electrophoresis of all the above mentioned samples was performed according to Hames (1990) with slight modifications (11). The 5% stacking gel was prepared in 120 mM KOH and 0.75% acetic acid (pH-5.9). Ammonium per sulfate (APS) and TEMED concentration was 0.7% and 0.06% respectively. 15% resolving gel was prepared in 30 mM KOH and 13.25% acetic acid (pH-2.9). The concentration of APS was similar to that of stacking gel. TEMED concentration was increased to 0.6%.Electrode buffer was 0.16% acetic acid containing 0.65% β-alanine, pH-2.9. Loading buffer contained 0.8% glycerol, 2% methylene blue, 120 mM KOH and 0.75% acetic acid (5.9). Samples were mixed with equal volumes of loading buffer for application onto the gel. Electrophoresis was performed in the cold (4°C) at 200V for 75 min. Gel was stained in coomassie blue stain (0.4% dye made in 50% methanol/10% acetic acid). Destaining was carried out in 30% methanol/10% acetic acid solution.

Two-dimensional electrophoresis (2-DE)

2-DE analysis was carried out according to O'Farrell (28). For first dimension 300µg of native and denatured ricin samples (boiled for 30 min at 100°C) in 130µl of sample rehydration buffer (8 M urea, 2% w/v CHAPS, 15 mM DTT and 0.5% v/v IPG buffer pH 3-10) was used to rehydrate IPG strip (7 cm, pH 3-10). The isoelectric focusing (IEF) was performed using immobilized pH gradient (IPG) strips in IEF unit (Bio-Rad, USA). Rehydration was done overnight and then focusing at 8000 VHr at 20°C under mineral oil. After the first dimension, the strips were equilibrated for 15 min in 50 mM Tris/HCL buffer, pH 8.8 containing 6 M urea, 30% w/v glycerol, 2% w/v SDS and 1% w/v DTT and then for 10 min in the same buffer containing 4% w/v iodoacetamide instead of DTT. After equilibration, strips were transferred to 12 % SDS-PAGE for second-dimension separation. Gels were stained with Coomassie Blue G-250.

Antiricin antibody preparation

Polyclonal antibody was raised against ricin. Purified preparation was converted to its toxoid by treating in boiling water bath. The rabbit was used as animal model for raising antibodies. The rabbit was immunized with toxoid and test bled to assess immune status (9). The antibody titer was determined by Octerlouny's double immune diffusion method and by Dot ELISA (10).

Western blot analysis

Western blot analysis was performed as described by Chaponi and Migliorini (2). Native and denatured ricin sample was separated on acidic gel and electrophoretically transferred onto PVDF membrane, (0.45 µm, pore size, Pierce Biotechnology, USA) using 0.7% acetic acid. Blotting was performed at constant voltage (80V) for 1.5 hr in Bio-Rad Trans-Blot apparatus. Temperature was kept low by using ice block supplied with the system.

For immunodetection, the blots were blocked for 2h with 5% low fat milk powder in blocking buffer (5% milk powder, 0.05% Tween-20 in PBS) at 37°C. Blots were washed 3 x for 15 min each with PBST (PBS containing 0.05% Tween-20) and then incubated with primary antibody at 1:1000 dilution overnight at 4°C. The blots were incubated with a secondary antibody, goat anti-rabbit IgG-HRP conjugate (1:50,000) for 90 min at 37°C after washing thrice with PBST. After another three washes, the blots were developed using an enhanced chemiluminescent detection system (Super Signal West Pico Chemiluminescent Substrate, Pierce) according to manufacturer's protocol and the images were taken on Pierce CL-XPosure™ X-ray film.

MALDI-TOF/MS

Mass spectrometric analysis was performed using MADLI-TOF instrument (Bruker Microflex LRF-20, Flex Control Workstation, Bremen, Germany) equipped with delayed extraction and a UV ionization laser (N_2, 337nm) with a 3-ns pulse width. The accelerating voltage was 20 Hz and the grid voltage was set to 18.3 kV. Protein molecular weight determination was carried out in positive ion linear mode. Hundred laser shots were averaged per spectra. For the peptide mass fingerprinting, the instrument was operated in the reflector mode and Five hundred laser shots were averaged per spectra. Protein Calibration standards include mixture of protein A (MW=22,307), trypsinogen, (MW=23,982), Protein A (MW=44,613) and BSA (MW=66,431). Peptide Calibration standards include mixture of angiotensin II, angiotensin I, substance P, bombesin, ACTH clip 1-17, ACTH clip 18-39 and somatostatin 28 covering the *m/z* range of 500-5000. The spectra were evaluated using the Flex Analysis Software (Bruker Daltonics). The MS spectrum obtained was submitted to MASCOT search via Bio tools version 3.1. The search parameters used were partial methionine oxidation, one missed cleavage, Peptide mass Tolerance 100 ppm and the database selected was NCBI /Swiss Prot.

MALDI-TOF/MS of purified protein

For routine MALDI analysis of purified protein, dried droplet method was used. The protein sample (1µg) was applied to the MALDI plate and was allowed to dry. Once dried an equal volume of MALDI matrix (sinapinic acid) was applied and allowed to dry at room temperature. The spectra were analyzed for determination of molecular weight of pure protein.

MALDI-TOF/ MS analysis of trypsin digested proteins

For in-gel trypsin digestion, the coomassie-stained protein band was excised and washed thrice with 400µl destaining solvent (50% v/v ACN in 25 mM NH_4HCO_3) with constant vortexing of 10 min. The washed gel pieces were dehydrated with 200µl of 100% ACN and dried in speed vac centrifuge. The protein contained in the gel was subjected to digestion using 25µl (20µg/100µl) of trypsin in 50 mM NH_4HCO_3 at 37°C for overnight in an incubator. The peptides were extracted twice from the gel using 200µl of extraction solvent (50% ACN, 5% TFA). This solvent was allowed for lyophilization for complete solvent removal. After lyophilization only a small pellet was remained which was used for mass analysis. The peptide extract was spotted on MALDI plate in addition with matrix. The matrix was prepared as a saturated solution of CHCA in deionized water-acetonitrile (1:1) containing 0.1%TFA. For complete purification, the dissolved peptides were eluted using ZipTip C18 (a Millipore product).

The in-solution trypsin digestion of ricin was carried out using 50µg of protein and 10µl of trypsin (20µg/100µl). The mixture was incubated at 37°C for overnight for complete digestion. From the digested sample, 1µl was spotted on MALDI plate and then 1µl of matrix (CHCA) was applied on the peptide spot.

Enzymatic deglycosylation of ricin and its subunits

Deglycosylation of ricin and its subunits was carried out using an enzymatic N-deglycosylation kit (GlycoProfile I-PP0200, Sigma) in accordance with the manufacturer's instructions. The kit includes peptide N-glycosidase F (PNGase F) and trypsin enzymes necessary for N-linked deglycosylation and tryptic digestion, respectively. The samples were desalted and concentrated for analysis by MALDI-TOF/MS with subsequent database searching. Thirty micrograms of protein from SDS-PAGE gel was deglycosylated following the protocol suggested by the manufacturer. The band was excised, destained and digested with 15µl of PNGase F overnight at 37°C. The PNGase F digested extract was collected. The glycopeptides were extracted thrice from the gel using 200µl of ultrapure water and pooled with the digested extract. The extract was lyophilized and retained for glycan analysis. The gel piece was dried and further digestion was done with 20µl of trypsin by overnight incubation at 37°C. The peptides were extracted from the gel as earlier described and used for MALDI-MS analysis.

Detection of denatured ricin in food samples

Apple juice (1ml) was spiked with denatured ricin (boiled for 10 and 15 min) at concentration of 50µg/ml. The samples were loaded on acidic native PAGE and ricin detection was made by staining with commassie. The bands were excised, trypsin digested and analyzed by MALDI-TOF/MS.

1ml of milk solution was spiked with 25µg of ricin and boiled for 15 min in boiling water bath. The spiked milk sample was precleared with 100µl of protein G- Sepharose beads (Santa Cruz) by incubation at 4°C for 3h with gentle rotation. An aliquot of 9µl of ricin antibodies in 191µl of HEPES buffer (50 mM, pH 7.3) was incubated on 100 µl of protein G beads for 60 min at 4°C. The protein G-antibody complex is added to precleared milk sample and further incubated overnight at 4°C. The beads with bound immune complexes were collected by centrifugation (4°C) at 3000 rpm and washed twice with 500µl of HEPES buffer to remove non-specifically bound species and, then three times with 500µl of ultrapue water. Ricin elution was accomplished by incubating the beads with 40µl of 0.1% TFA for 30 min at room temperature. The supernatant was collected and its pH was neutralized by adding 5µl of 200mM ammonium bicarbonate. It was lyophilized in a vacuum centrifuge and subjected to digestion with 35µl of trypsin overnight at 37°C. The digested sample was again lyophilized and used for mass analysis.

In vivo toxicity studies

Swiss albino male mice weighing between 25 and 30 gm, bred at Defence Research and Development Establishment (DRDE) animal facility were used in this study. The animals were kept at room temperature with 12 h light/dark cycles. The animals were fed on standard pellet diet (Aashirwad Ltd, Chandigarh), and were maintained on dust free rice husk bedding in polypropylene cages. Food and water were given ad libitum. The animals were handled according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on animals (CPSEA, India) and the experiment was approved by Institute ethics committee on animal experiments. Control mice were injected with milk heated for 15 min. The treated group was injected 20µg/kg body weight from heat treated milk sample spiked with ricin through intraperitoneal (i.p.) route. Native ricin in heat treated milk was injected at the dose of 20µg/kg body weight to animals. The animals were observed for 7 days and the data were collected on mortality and body weight.

RESULTS

Purification and partial characterization of ricin and its subunits

Ricin was purified from the seeds of castor plant to apparent homogeneity by gel filtration chromatography on Bio gel A 0.5m column. The elution profile of purified ricin is shown in the Figure 1a.

The A- and B-chains were separated by affinity chromatography on lactamyl sepharose 4B in the presence of 0.5 M $MgCl_2$ and 0.01 M DTT. As shown in the chromatogram (Figure 1b), A-chains passed through the column during washing of the column with 0.05 M Tris-HCl (pH 8.5) containing 0.5 M $MgCl_2$, 0.01 M DTT while the B-chain bound to the lactosyl residues was further eluted with the same buffer supplemented with 0.4 M lactose. The amounts of chains recovered were determined by UV absorbance and the yields were 50% for A- and 50% for B chain.

The purity of ricin was evaluated by SDS-PAGE. The relative migration rate of ricin under reducing and non-reducing conditions to those of molecular weight markers has been presented in Figure 2a. Under non-reducing condition, ricin gave a single band in 60-65 kDa region. When ricin was treated with β-mercaptoethanol, it gave

two bands in the region of 30-34 kDa region which represent A and B subunits of the ricin.

When analyzed by SDS-PAGE, the purified A subunit of affinity purified ricin showed two molecular mass variants which may be of differential glycosylation. The A chain fraction revealed one major and one minor band in 30-34 kDa region. The B-chain revealed one band (Mr=33-34 kDa) compared to the molecular weight markers separated on the same gel (Figure 2b).

SDS and acidic native PAGE of native and denatured ricin

Only native ricin appeared on SDS-PAGE in 60 kDa region (Figure 3a). Ricin denatured for different period cannot be detected indicating its degradation on boiling. Acidic native PAGE of native and denatured ricin gave evidence that ricin is degraded on boiling (Figure 3b). The band intensities of native and boiled ricin samples are different but their mobility was similar. It was also observed that as boiling time increases the degradation of ricin is also increased.

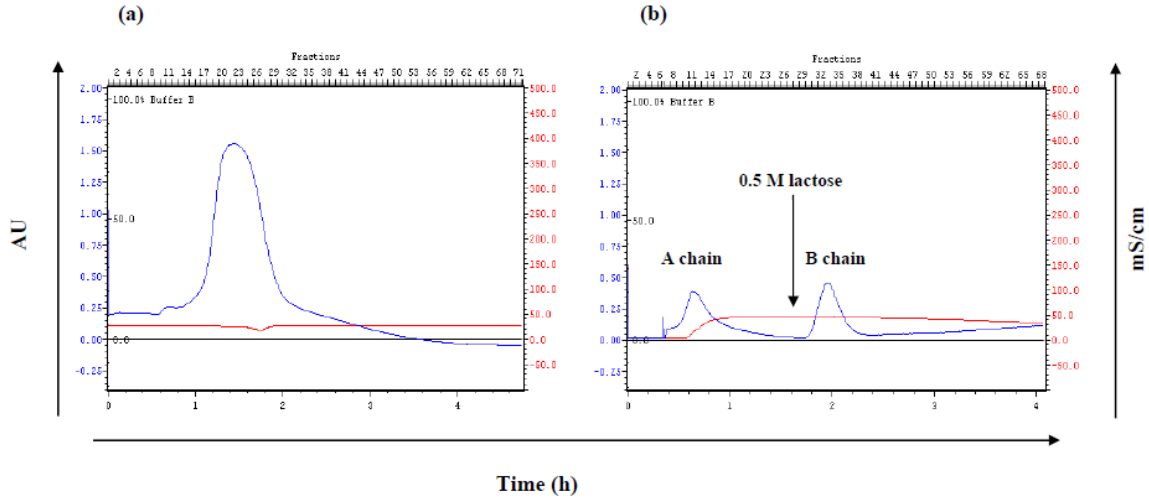

Figure 1. **(A)** Gel –filtration chromatography of crude ricin on Bio-gel A-0.5m column. The column was equilibrated with 0.05 M PBS, pH-7.2, containing 0.1 M NaCl. Crude ricin was passed through Biogel A 0.5m column and elution was carried out with the equilibration buffer. The flow rate was 1.0ml/min and 4ml of the effluent was collected per tube. Peak I corresponds to Ricin. **(B)** Separation of the ricin A-and B-chains on lactamyl-sepharose 4B matrix. Ricin (14.8 mg/1.25ml) in 0.05 M Tris-HCl, pH 8.5, was incubated with 0.05 M DTT for 3 hr at 37°C. After cooling, the reduced ricin was loaded on the column and 4ml fractions were collected. The flow rate was 0.75ml/min. Proteins were detected by uv absorbance at 280 nm. The A fraction was eluted with 0.05 M Tris-HCl, pH 8.5,0.5 M $MgCl_2$, 0.01 M DTT and the B fraction with the same buffer supplemented with 0.4 M lactose.

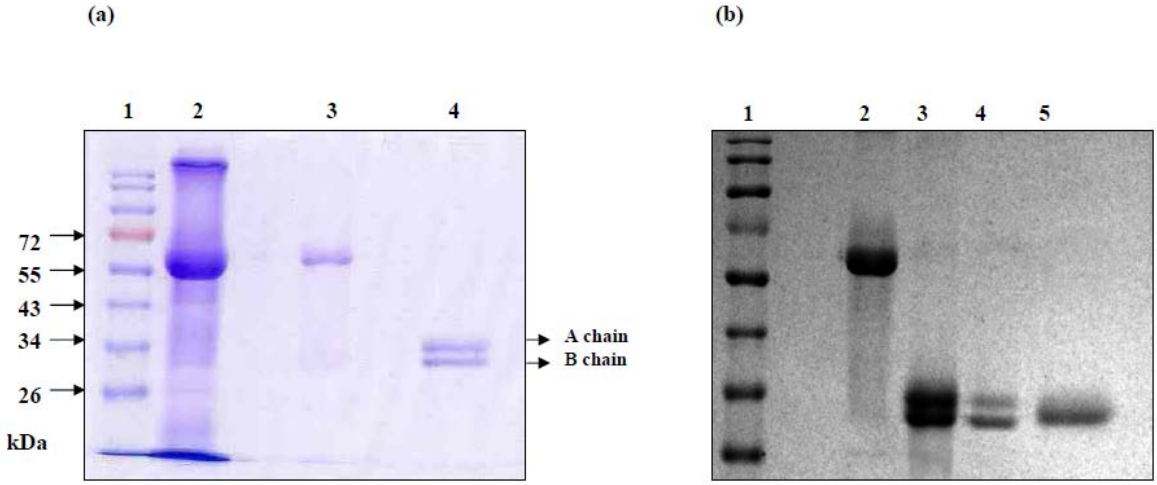

Figure 2. **(A)** SDS-PAGE of ricin on 12% acrylamide gel. Lanes: 1) Marker; Lane 2) Crude ricin; Lane 3) Purified ricin (non-reduced); Lane 4) Purified ricin in presence of β-mercaptoethanol (reduced). **(B)** SDS-PAGE of separated ricin A and B chains. Lanes: 1) Standard Marker; 2) Unreduced ricin (20 µg); 3) Reduced ricin (20µg); 4) A fraction (12µg); 5) B fraction (12µg).

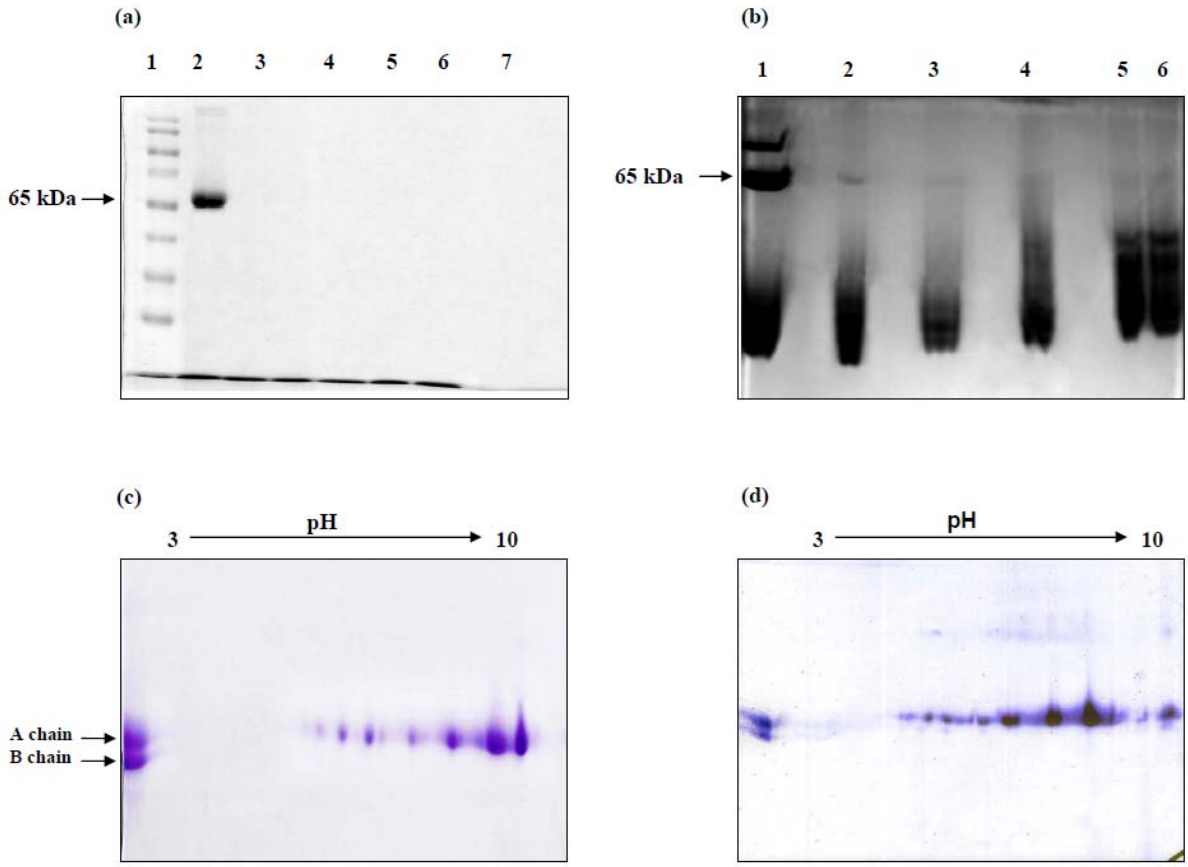

Figure 3. (A) SDS-PAGE and **(B)** acidic native PAGE of native and denatured ricin. (a) Lanes: 1) Standard Marker; 2) Native ricin ; 3) 3.75 denatured ricin; 4) 7.5 min denatured ricin; 5) 15 min denatured ricin; 6) 30 min denatured ricin; 7) 60 min denatured ricin. (b) Lanes: 1) Native ricin; 2) 3.75 min denatured ricin ; 3) 7.5 denatured ricin ; 4) 15 min denatured ricin; 5) 30 min denatured ricin; 6) 60 min denatured ricin. 2-D gel patterns of **(C)** native and **(D)** denatured ricin.

2-DE of native and denatured ricin

Native and denatured ricin was characterized by 2-D analysis. The observed changes in the protein patterns, displayed in the commassie-stained gels could be related to the events occurring in boiling. Figure 3c and 3d show 2-D gels of ricin before and after boiling. As expected, clear differences could not be made from the 2-D maps. However, a slight increase in the number of protein spots in the range of 30-34 kDa for ricin after boiling at 100°C for 30 min. was detected in 2-D patterns, indicating that the formation of a small amount of soluble aggregates via hydrophobic interactions.

Immunological analysis of native and denatured ricin

The western blots were carried out for both the samples at similar concentration to find out whether denatured sample still retained its immunological property. The native ricin could be detected after an exposure time of 10 min on X-ray film (Figure 4a), but detection of denatured ricin treated at 3.75 and 7.5 min require a longer exposure time of 90 min (Figure 4b) along with cleavage products. Ricin denatured for 15 min can be detected to some extent only after 180 min exposure (Figure 4c). The detection of ricin denatured for 30 and 60 min was not possible even after prolonging the exposure time suggesting a weak signal from ricin due to its complete degradation.

MALDI-TOF/MS of purified ricin and its subunits.

SDS-PAGE analysis showed ricin and its subunits have higher molecular mass than measured by MALDI-TOF/MS due to their difference in migration pattern. The MALDI spectra of ricin A chain, B chain and purified ricin in linear positive ion mode using sinapinic acid as matrix are shown in Figure 5 a, b and c.

Two distinct peaks, singly (*m/z* 62,821.8) and doubly (*m/z* 31,498.3) protonated molecular ricin ions were detected on the mass spectrum of ricin. The mass spectrum of ricin A chain revealed two distinct peaks for different A chain variants whereas a single peak was observed for ricin B chain. The first peak was identified as RTA_1 and the second peak corresponded to RTA_2. The molecular mass of two RTA (RTA_1 and RTA_2) variants corresponded to 31,325 Da, 32,596 Da respectively and 31,946 Da for B chain. The MALDI mass spectra of denatured ricin has showed an intense peak of 11,113 Da which might be due to the presence of polypeptide chain of nearly 100 amino acids cleaved from the C-terminal and N-terminal sides of A chain of native ricin during boiling (Figure 5d).

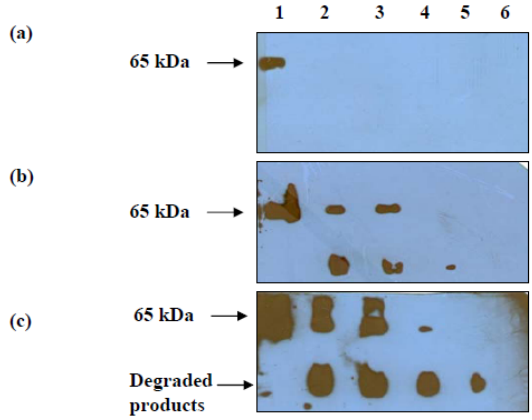

Figure 4. Western blots of native and denatured ricin X-ray film exposed for **(A)** 10 min; **(B)** 90 min; **(C)** 180 min. Lanes: 1) Native ricin; 2) 3.75 min denatured ricin; 3) 7.5 denatured ricin ; 4) 15 min denatured ricin; 5) 30 min denatured ricin; 6) 60 min denatured ricin.

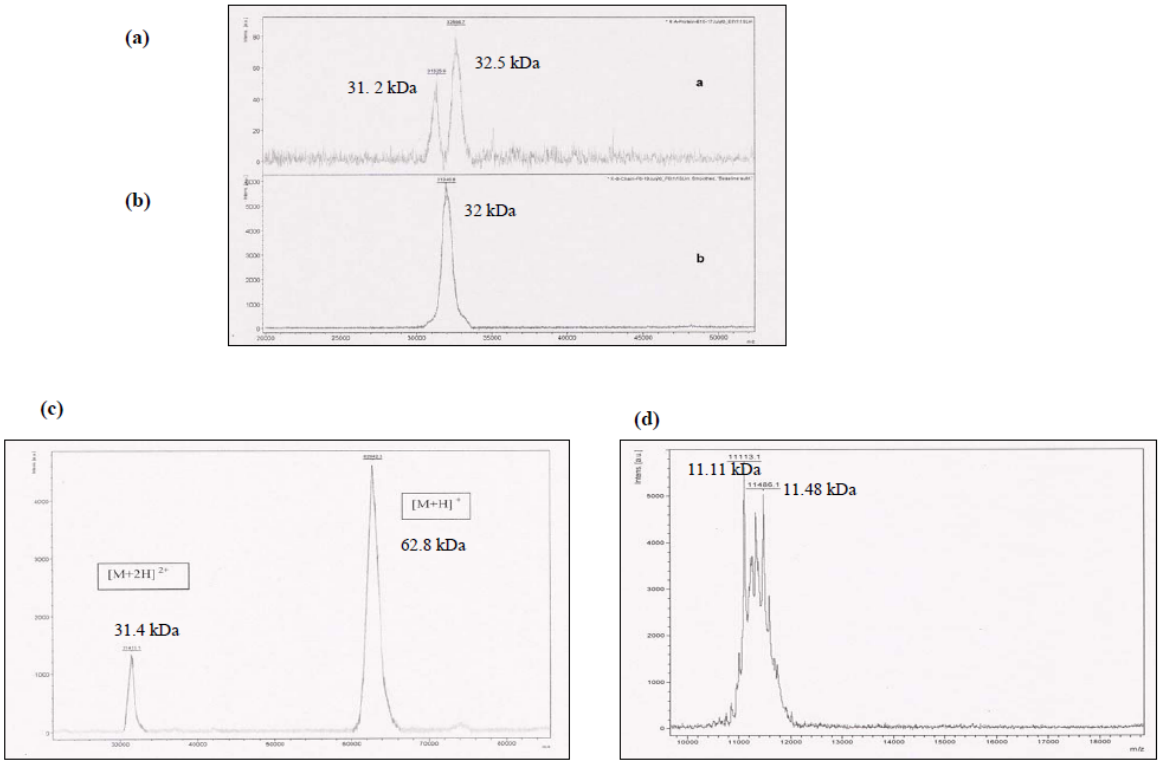

Figure 5. MALDI-TOF Mass spectrum of ricin and its sub units. **(A)** A (A_1 & A_2) chain; **(B)** B chain showing molecular mass at 31.32kDa, 32.59 and 31.94 kDa respectively in the linear mode using sinapinic acid as matrix; **(C)** MALDI –TOF mass spectrum of native ricin showing $[M+H]^+$ 62.8kDa and $[M+2H]^{2+}$ 31.4 kDa in the linear mode using sinapinic acid as matrix.; **(D)** MALDI–TOF mass spectrum of denatured ricin showing 11.11 kDa as the major peak in the linear mode using sinapinic acid as matrix.

MALDI-TOF/MS of trypsin digested ricin and its subunits

The tryptic peptide mass finger prints of ricin (Figure 6a and 6b), A and B chain were acquired using MALDI-TOF mass spectrometer in reflectron mode using CHCA matrix for both in-solution and in-Gel digests. The basic sequence of ricin is given in Figure 7. The trypsin digested ricin A chain and B chain gave multiple peptide peaks which is shown in Table 1. We were able to detect twenty four peptides with the help of Mascot algorithm (www.matrixscience.com). These peptides showed significant sequence homology to the ricin. The Mascot

database has identified 13 peptides for A chain and 11 peptides for B chain which has gave the sequence coverage of about 63% of ricin (Table 2). The three selected marker peptides - *m/z* 1013.6, *m/z* 1310.7 and *m/z* 1728.8 were chosen for the identification of ricin in a sample as these were detected with highest sensitivity under MALDI-TOF/MS conditions and were highly conserved in their sequence. It was further confirmed by the post source decay (PSD) analysis of the 3 marker peptides by selecting precisely the parent ion and performing MS/MS analysis. These peptides were selected with fragment mass tolerance of 100 ppm and searched for MS/MS sequence using Swiss Prot database in Viridiplantae taxanomy which gave the sequences 161-LEQLAGNLR-169, 150-YTFAGGNYDR-160, 233-SAPDPSVITLENSWGR-248 respectively. Along with the marker peptides, other intense ion of *m/z* 2259.3 was subjected to PSD analysis which gave the sequence 214-LSTAIQESNQGAFASPIQLQR-234 and identified unequivocally ricin by MS/MS Mascot search.

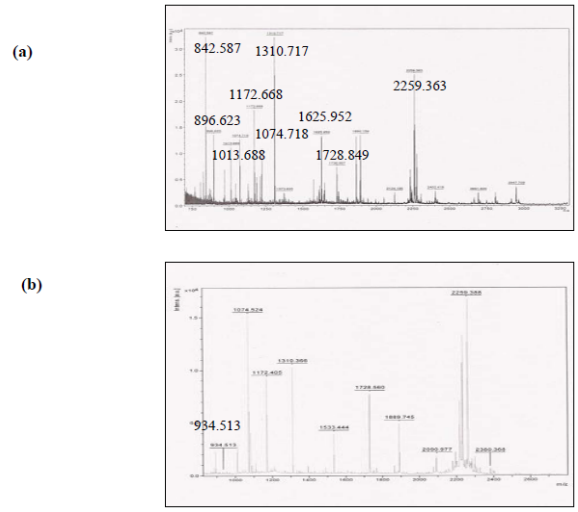

Figure 6. Peptide Mass Finger Print of ricin. In-gel tryptic digest of **(A)** native and **(B)** denatured ricin in the reflectron mode using CHCA as matrix.

TSS (35) - A- Chain (267) aa- 12 aa Linker peptide- B-Chain (262) aa

TSS (Terminal Signal Sequence): MKPGGNTIVI WMYAVATWLC FGSTSGWSFT LEDNN

A-Chain IFPKQYPIINFTTAGATVQSYTNFIRAVRGRLTTGADVRHEIPVLPNRVLPINQR
FILVELSNHAELSVTLALDVTNAYVVGYRAGNSAYFFHPDNQEDAEAITHLFTDVQNRYTFAFGGNYDR
LEQLAGNLRENIELGNGPLEEAISALYYYSTGGTQLPTLARSFIICIQMISEAARFQYIEGEMRTRIRYNRRS
APDPSVITLENSWGRLSTAIQESNQGAFASPIQLQRRNGSKFSVYDVSILIPIIALMVYRCAPPP SSQF
Linker Peptide: SLLIRPVV PNFN
B-Chain: ADVCMDPEPIVRIVGRNGLCVDVRDGRFHNGNAIQLWPCKSNTDANQLWTLKR
DNTIRSNGKCLTTYGYSPGVYVMIYDCNTAATDATRWQIWDNGTIINPRSSLVLAATSGNSGTTLTVQTNI
YAVSQGWLPTNNTQPFVTTIVGLYGLCLQANSGQVWIEDCSSEKAEQQWALYADGSIRPQQNRDNCLTSD
SNIRETVVKILSCGPASSGQRWMFKNDGTILNLYSGLVLDVRASDPSLKQIILYPLHGDPNQIWLPLF
Amino Acid Composition
A41 C12 D26 E21 F21 G41 H6 I44 K10 L50 M8 N43 P31 Q29 R35 S43 T42 V36 W13 Y24

Figure 7. Sequence of Ricin.

Table 1: Tryptic peptides fromp Ricin identified by MALDI-TOF/MS.

T.No.	Position	Peptide Sequence	Measured masses $[M+H]^+$	Theoretical masses $[M+H]^+$
	A Chain			
T1	1-26	(-) IFPKQYPIINFTTAGATVQSYTNFIR.A	2990.8510	2989.8437
T2	30-39	R.GRLTTGADVR.H	1045.6738	1044.5676
T3	32-48	R.LTTGADVRHEIPVLPNR.H	1887.7654	1886.7531
T4	40-48	R.HEIPVLPNR.V	1074.7175	1073.5982
T5	49-56	R.VGLPNQR.F	896.6225	895.5239
T6	86-114	R.AGNSAYFFHPDNQEDAEAITHLFTDVQNR.Y	3307.6541	3306.496
T7	115-125	R.YTFAFGGNYDR.L	1310.7170	1309.5728
T8	115-134	R.YTFAFGGNYDRLEQLAGNLR.E	2304.7489	2303.7746
T9	126-134	R.LEQLAGNLR.E	1013.6884	1012.5665
T10	181-189	R.FQYIEGEMR.T	1172.6683	1171.5322
T11	198-213	R.SAPDPSVITLENSWGR.L	1728.8491	1727.8418
T12	214-234	R.LSTAIQESNQGAFASPIQLQR.R	2259.3627	2258.1655
T13	239-265	K.FSVYDVSILLPIIAMVYRCAPPPSSQ (-)	3013.9040	3012.8967
	B Chain			
T14	1-12	(-) ADVCMDPEPIVR.I	1345.5889	1344.6286
T15	17-24	R.NGLCVDVR.D	876.0282	875.4404
T16	28-40	R.FHNGNAIQLWPCK.S	1528.7867	1527.7525
T17	41-52	K.SNTDANQLWTLK.R	1390.6289	1389.6216
T18	41-53	K.SNTDANQLWTLKR.D	1547.7213	1546.7972
T19	63-89	K.CLTTYGYSPGVYVMIYDCNTAATDATR.W	2967.3425	2965.3053
T20	90-102	R.WQIWDNGTIINPR.S	1613.8274	1612.8213
T21	169-187	K.AEQQWALYADGSIRPQQNR.D	2232.4352	2231.0952
T22	204-245	K.ILSCGPASSGQR.W	1176.3488	1175.5837
T23	220-236	K.NDGTILNLYSGLVLDVR.A	1863.1339	1862.0018
T24	244-262	K.QIILYPLHGDPNQIWLPLF (-)	2278.7193	2277.2430

Table 2. Mascot database search for Ricin and its subunits.

Protein	No. of amino acids	No. of peptides matched	Sequence coverage	Mascot Score	pI
Ricin	576	24	63 %	207	6.34
A chain	267	13	78 %	125	6.49
B chain	262	11	63 %	-	5.70

Table 3. Denatured Ricin detection by MALDI-TOF/MS.

Peptide	Sequence	Native Ricin $[M+H]^+$	Boiled Ricin $[M+H]^+$
A-32-39	LTTGADVR	832.478	832.478
A-49-56	VGLPNQR	896.622	896.550
A-259-267	CAPPPSSQF (-)	--------	934.513
A-126-134	LEQLAGNLR	1013.688	1013.635
A-40-48	HEIPVLPNR	1074.717	1074.524
A-181-199	FQYIEGEMR	1172.668	1172.405
A-115-125	YTFAFGGNYDR	1310.717	1310.366
A-198-213	SAPDPSVITLENSWGR	1728.849	1728.560
A-32-48	LTTGADVRHEIPVLPNR	1887.765	1889.745
A-214-234	LSTAIQESNQGAFASPIQLQR	2259.362	2259.388
A-1-26	(-) IFPKQYPIINFTTAGAT VQSYTNFIR	2990.851	2991.442

Ricin was denatured in boiling water bath for different time intervals of 3.75, 7.5, 15, 30 & 60 min and subjected to in-solution trypsin digestion. The PMF of denatured ricin showed 11 peptide fragments, irrespective of the boiling time as similar pattern was observed in all the samples (Table 3). Five peptides fall in 1-100 amino acids of A-Chain which is also shown by the 11.11 kDa peak in the boiled ricin analysis. These five peptides resulting from the tryptic digest of 11.11 kDa peak and lying in 1-100 amino acids of A chain showed complete match with the theoretical digest of A chain also. The three marker peptides were observed in all the samples revealing ricin identification and also confirming that these peptides were not lost during boiling and conserved in their sequence. The differentiating peptide (*m/z* 934.513) was observed in denatured ricin as a result of cleavage of disulfide bond (S-S) between 294 and 318. The same was not seen in native ricin because of its involvement in inter-chain disulfide bond at position 294-318.

MALDI-TOF/MS of deglycosylated ricin and its subunits

To study and determine glycosylation sites in ricin and its subunits, they were deglycosylated using an enzymatic deglycosylation kit. The resulting glycopeptides were analyzed in the reflectron mode and submitted for search in ExPASy-Glycomod tool. Nearly twelve glycopeptide fragments with basic peptide sequence of NGSK (*m/z* 404.20193) at position 271-274 in ricin were obtained (Table 4). Ricin contains four potential-N-glycosylation sites, Asn 10 and Asn 236 (position 271-274 in ricin molecule) for A-chain, and Asn 95, Asn 135 for B chain (5). Although several probable structures were identified for the glyocsylation at Asn-236 in the A-chain, only one glycopeptide was observed for the glycosylation at Asn-95 in B-Chain. The Glycopeptides at other sites could not be observed as they were outside the monitored m/z range. Glycopeptides bearing Asn 236 in A-Chain were mainly hybrid/complex type with mannose as hexose sugar units.

MALDI-MS detection of denatured ricin in apple juice and milk

Detection and identification of ricin was further confirmed by the presence of ricin marker peptides in the mass spectrum of the food samples. We were able to detect denatured ricin from apple juice and milk samples when spiked with toxin at 50µg/ml of juice and 25µg/ml of milk by acidic native PAGE (Figure 8a and 8b) and immunocapture respectively. Further, we were able to detect ricin in its inactive form which is demonstrated as non toxic in mice.

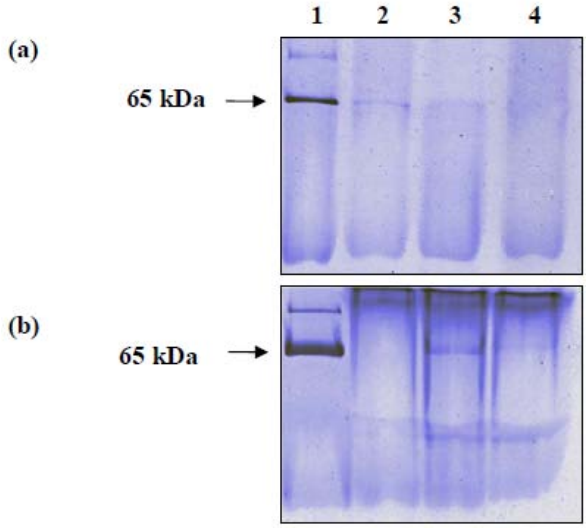

Figure 8. Acidic native PAGE of spiked (A) apple and (B) milk juice for detection of denatured ricin. (a) Lanes: 1) Native ricin; 2) 10 min denatured ricin; 3) 15 min denatured ricin; 4) Control (apple juice). (b) Lanes: 1) Native ricin; 2) Control (milk); 3) 10 min denatured ricin; 4) 15 min denatured ricin.

In vivo toxicity of denatured ricin in milk

There is significant difference in toxicity of native and heat denatured ricin. Table 5 represents toxicity comparison of native and denatured ricin. No mortality was observed in case of animals treated with heat treated milk spiked with ricin. Treated animals also didn't show any significant change in body weight for a period of seven days. Ricin at the dose of 20µg/kg body weight was lethal to the animals.

DISCUSSION

Ricin, a phytotoxin was extracted and purified from the Ricinus communis seeds to apparent homogeneity by gel filtration chromatography. The purified ricin showed a single band in 60-65 kDa region under non-reducing condition and two bands in 30-34 kDa region under reducing condition when analyzed by SDS-PAGE. Ricin A and B chain was also separated by affinity chromatography and their elution profile was very much similar to the pattern obtained by Emmanuel et.al (6). Ricin having molecular weight from 60,000 Da to 65,000 Da has been reported by SDS-PAGE

Table 4. Glycopeptides determination in Ricin by MALDI-TOF/MS.

Glycoform mass	Probable structure type	Peptide	Peptide sequence	Theoretical glycopeptide mass $[M+H]^+$	Measured glycopeptide mass $[M+H]^+$
A Chain					
511.190	$(Hex)_1(HexNAc)_1(Deoxyhexose)_1$	404.202	271-274 NGSK	916.399	916.412
670.243	$(HexNAc)_2(Pent)_2$	404.202	271-274 NGSK	1075.452	1075.529
684.259	$(HexNAc)_2(Deoxyhexose)_1(Pent)_1$	404.202	271-274 NGSK	1089.468	1089.496
689.238	$(Hex)_3(HexNAc)_1$	404.202	271-274 NGSK	1097.997	1097.981
733.228	$(Hex)_1(NeuGc)_1(Pent)_2$	404.202	271-274 NGSK	1138.437	1138.402
760.239	$(Hex)_1(NeuAc)_1(NeuGc)_1$	404.202	271-274 NGSK	1165.448	1165.640
855.241	$(Deoxyhexose)_1(Pent)_2(Phos)_1$	404.202	271-274 NGSK	1260.450	1260.616
906.297	$(HexNAc)_1(NeuGc)_1(Pent)_3$	404.202	271-274 NGSK	1311.506	1311.616
945.236	$(Hex)_1(Pent)_3(NeuGc)_1(Phos)_1$	404.202	271-274 NGSK	1350.445	1350.634
979.313 1384.710	$(Hex)_1(HexNAc)_1(NeuGc)_2$	404.202	271-274 NGSK	1384.522	
1288.444	$(Hex)_3(HexNAc)_2(Pent)_3$	404.202	271-274 NGSK	1693.653	1693.772
2429.776	$(HexNAc)_2(Deoxyhexose)_1(NeuAc)_1$ $(NeuGc)_1(Phos)_1+(Man)_3(GlcNAc)_2$	404.202	271-271 NGSK	2834.529	2834.985
B Chain					
788	$(Hex)_1(HexNAc)1(NeuAc)_1(Pent)_1$	1611.81	404-416 WQIWDNG-TIINPR	2399.810	2401.090

Table 5. Toxicity comparison of native and denatured Ricin (spiked in milk).

Group	Dose of ricin	No. of animals dosed	No. of animals died	7-day body weight
Control (Heat treated milk)	------	4	0	111.62 ± 1.8
Denatured ricin (Spiked and heat treated in milk)	20μg/kg	4	0	124.13 ± 1.3
Native ricin (Spiked in heat treated milk)	20μg/kg	4	4	------

technique (27). Under reducing condition, ricin appeared to have two subunits, corresponding to the molecular masses of 30 kDa and 32 kDa (39). Ricin can also be separated at pH-2.9 by acidic PAGE. Ricin and RCA differ in their electrophoretic mobility at acidic pH (2.9). Electrophoretic mobility of ricin is higher than that of RCA and therefore can be resolved on acidic gel (24).

Considering the recent situations involving biological warfare terrorism, the detection of ricin at picomolar level using complementary analytical methods is of immense toxicological requirement. Mass spectrometric methods are useful to achieve rapid, sensitive and highly specific characterization of proteins. In this study native and heat denatured ricin were characterized by MALDI-TOF/ MS. The tryptic digest of ricin and its subunits was completely characterized and the sequence coverage of ricin was found to be 63 %. MALDI-TOF/MS revealed two peaks for ricin A chain which may be due to difference in their carbohydrate content (19). Their molecular mass was found to be similar to those reported by Na et. al (26). We were able to determine few glycopeptides in ricin molecule which may be the basis of biovariation in its structure. The potential glycosylation site was identified in ricin A chain at Asn 236. The mass difference (1271 Da) between RTA_2 & RTA_1 is due to the higher carbohydrate content in RTA_2 which contained high mannose linked to Asn-236. It can be concluded that this differential glycosylation at asparagine units might be responsible for the existence of ricin variants.

The literature contains many references to inactivation of ricin during thermal treatment of whole or flaked castor seeds or castor bean meal (15, 29). Jenkins (15) reported that by boiling solutions of ricin for 2 min fully detoxified ricin as measured in a rat feeding study. This study corroborates our finding that heating ricin at 100°C resulted in a significant loss of toxicity. This could be due to the formation of ricin complexes at high temperatures (13). The complex could be formed by self-aggregation of ricin, binding with other proteins, or inorganic ions in the food matrix. We were able to detect and differentiate native (toxic) and denatured (non-toxic) ricin by mass spectrometry. MALDI-MS can be used to prove the intact ricin and to screen samples for ricin peptides. The three markers peptides were chosen for the detection of ricin in a sample. In addition to this, we have developed a novel method that can detect denatured ricin in spiked food samples by using antibody-capture followed by tryptic digestion and mass spectrometry detection of ricin specific peptides. This shows that denatured ricin preserved the immunobinding ability. We were able to obtain only one differentiating peptide maker for denatured ricin. The identification of such markers in heat treated ricin samples is essential for differentiating native and denatured

ricin. Preparation of marker peptide library by advanced analytical techniques LC-ESI/MS and MS-MS will provide immense potential to this study. No B-chain peptides were seen in any of the digest sample in this study suggesting its complete degradation during boiling which may be due to more number of intra-chain disulfide bonds. It has been reported earlier that the proteins with a different number of cysteine residues can undergo degradation of their disulfide bonds at 100°C in the physiologically and biotechnologically relevant pH range (from 4 to 8) (36).

As denatured ricin can be used in place of native ricin as a threat, so this study will be helpful to find some specific measures to overcome the panic in the civilian population. Thus, this piece of work will be useful for the alleged non-adherence of CWC by Organization for the prohibition of Chemical weapons (OPCW).

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