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TECHNICAL NOTE

TOXICOLOGY

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Denatured Ricin Can Be Detected as Native Ricin by Immunological Methods, but Nontoxic *In Vivo*

ABSTRACT: Ricin is a glycoprotein from *Ricinus communis* seeds. It is known to have diverse toxic effects on cells of different visceral organs. In the present study, we purified and denatured ricin in a boiling water bath for different time intervals. We further made an attempt to identify native and denatured ricin by immunobased detection systems. All the antigen/antibody-based assays identified native and denatured ricin. On SDS–PAGE, only native ricin was observed. In western blotting, ricin boiled for 3.75 min gave a strong band on X-ray film. On native polyacryl amide gel electrophoresis, native and denatured ricin gave ricin band in 60-kDa region. The denatured ricin did cause mortality up to 25 mg/kg, while 5 and 10 μg/kg of native ricin caused 50% and 100% mortality, respectively. Detection of native and denatured ricin is very difficult, and the investigating agencies, forensic scientists, and analysts should be very careful while interpreting the results.

KEYWORDS: forensic sciences, ricin, denatured ricin, Dot ELISA, ICT, toxicity, SDS–PAGE

Ricin is an extremely toxic plant protein with a molecular weight of 65 kDa. It can be isolated from seeds of *Ricinus communis*, commonly known as castor seeds (Fig. 1). The castor plant grows globally. The global castor seed production is around 1 million tons per year. Castor seeds contain *c.* 40–50% oil. The castor oil has numerous applications in transportation, cosmetics, pharmaceutical, and manufacturing industries. The castor cake is one of the most versatile natural manures (1). The castor seed cake also contains highly toxic toxin ricin. Crude ricin can be isolated from defatted castor cake (residue remains after oil extraction) after extraction of cake at acidic pH. The crude ricin is further purified using affinity and size exclusion chromatographies. The ricin content of castor seeds varies from 1.0% to 2.0% of whole seed (1).

FIG. 1—*Photograph of castor seeds (Source: http://en.wikipedia.org/wiki/Ricin).*

The ricin molecule is comprised of two glycoprotein chains A and B (Fig. 2) and joined by a disulfide bond (2). The B chain binds to galactose residues present on various cell-surface glycoproteins and glycolipids and triggers endocytosis of the toxin. The A chain reaches the cytosol through Golgi complex after the reduction of the disulfide bond. Ricin A chain exhibits an RNA *N*-glycosidase activity that hydrolyzes a specific adenine residue present at 4324 position from a highly conserved loop region of 28S rRNA (3,4). This RNA *N*-glycosidase activity results in loss of protein elongation and presumably the subsequent death of exposed cell because of protein synthesis inhibition. Ricin endocytosis may also occur through another recognized process that involves the interaction of mannose-containing carbohydrate side chains of the toxin with mannose receptors. The mechanism of ricin toxicity may include ribosome inactivation, disturbance in calcium–magnesium balance, release of cytokines, acute phase reactions, oxidative stress in the liver, and apoptosis (5). Ricin or ricin A chain has been used to synthesize immunotoxins that show specific anticancer and anti-AIDS activities *in vitro* and *in vivo* (6).

Because of the extreme toxicity of ricin, there is a possibility that it can be misused as a chemical warfare agent (schedule I of chemical warfare chemical) or by terrorist organizations. In the past, ricin has been used for suicidal, homicidal, and malicious activities (7,8). In the recent past, a terrorist group was suspected to be producing ricin (9).

In the literature, various qualitative and quantitative methods are described for the detection of ricin. Most of them are immunology-based detection assays including enzyme-linked immunosorbent assay (10,11), radioimmunoassay (12), chemiluminescence immunosorbent assay (13), immuno-chromatography technique (ICT)-based assay (14), array biosensors (15), hydrogel-based protein microchips

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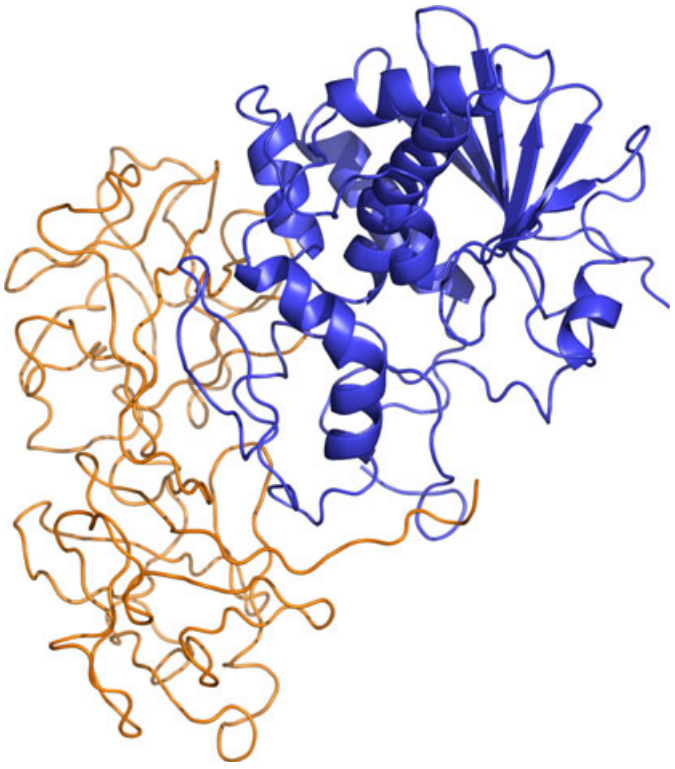

FIG. 2—*Structure of ricin. The A chain is shown in blue and B chain in orange (Source: http://en.wikipedia.org/wiki/Ricin).*

(16), and immuno-polymerase chain reaction (17). There is a possibility that antigen-based and antibody-based ricin assay can detect both native as well as denatured ricin. These assay procedures are not specific for native ricin as they can detect both native and denatured ricin and can cause confusion. Therefore, a very specific detection system is required that can differentiate native and denatured ricin. In the present investigation, we compared the detection efficiency of native and denatured ricin and also compared their *in vivo* toxicity.

Methods

Chemicals

Castor seeds were purchased from a local market. Defatted castor seed meal was prepared in house. Ricin was also prepared in house. Sepharose 4B was obtained from Sigma Chemicals (St. Louis, MO), and lactamyl sepharose affinity column was prepared in house. Latex bead (CLB-9), anti-rabbit IgG, horse radish peroxidase (HRP)-conjugate, diamino-benzedine (DAB), 4-chloro-1-naphthol, and all polyacryl amide gel electrophoresis (PAGE) reagents were purchased from Sigma Chemicals. Dot ELISA comb was purchased from mdi Membrane Technologies (Ambala, India). The ELISA plate was read by multidetection system, (Model Synergy HT, Biotek Instrument Inc., Winooski, VT), and special 96-well black polystyrene plates were used that were purchased from Costar (Cole-Parmar, IL). Tetracore® Ricin ICT card was a kind gift from Radix BioSolutions (Georgetown, TX). All other reagents used were of analytical grade.

Ricin Purification

Ricin was purified in the laboratory from *R. communis* seeds as described elsewhere (18). Briefly, the defatted castor seed meal was prepared in house. For the preparation of defatted castor seed meal, castor seeds were grinded in a grinder and treated with chilled ether for several times, till the complete removal of oil. Defatted castor seed meal was treated with 5% acetic acid and crude ricin was extracted. It was further purified in a single step by using lactamyl sepharose affinity column. The sepharose 4B was activated according to Hegde et al. (19) by introducing an epoxy group, amination of an epoxy activated gel, and subsequent coupling of ligand lactose. Under these conditions, the lectins bind to the column matrix, which were then eluted with 0.4 M lactose. The eluted peak was further subjected to gel filtration to separate on the basis of their molecular size. The fractions corresponding to peak were pooled, dialyzed extensively against PBS, and lyophilized. PAGE under reduced and nonreduced conditions was performed each time to assess the purity of ricin.

Preparation of Ricin and Animal Exposure for Toxicity Studies

Ricin was denatured in a boiling water bath for 3.75, 7.5, 15, 30, 60, and 120 min. After boiling for particular time period, samples were cooled at room temperature. Swiss albino male mice randomly bred in the Institute's animal facility, weighing between 30 to 35 g were used in this study. The animals were maintained on standard conditions of humidity, temperature (25 ± 2°C), and with 12 h light/dark cycles. Sterilized and dust-free rice husk was used as bedding in polypropylene cages. The animals were fed standard pellet diet (Ashirwad Brand, Chandigarh, India), 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 (CPCSEA, India), and the experiment was approved by Institute ethics committee on animal experiments. The mice were injected 25 mg/kg body weight from each boiled sample through intraperitoneal (i.p.) route. Native ricin was also injected at the dose of 2.5, 5.0 10, and 20 μg/kg body weight to control animals. The animals were observed for 14 days and data were collected on mortality and body weight.

Antiricin Antibody Preparation

The polyclonal antiricin serum was raised in rabbits in house using formalinized ricin toxoid (20) and IgG was purified by using Bio-Rad Econo-Pac serum IgG Purification kit (Bio-Rad, Hercules, CA). The ricin toxin was incubated with 1% formalin at 37°C for 21 days. The first dose was given in complete Freunds adjuvant and subsequent doses in incomplete Freunds adjuvant. After booster dose, animals were bleeding and antisera were collected. The antisera were further subjected to IgG purification using Bio-Rad IgG purification kit as per manufacturer's instruction. Briefly, the column was filled with agarose gel covalently cross-linked with Cibacron Blue. The serum was first passed through the desalting column (Econo-Pac 10 DG column). The serum sample was eluted by using 4 mL of application buffer provided with the kit. The 4 mL of sample collected was then passed through the Econo-Pac serum IgG purification column.

Column Chromatography of Native and Boiled Ricin

The column chromatography was performed on Bio-Rad, medium pressure liquid chromatography (MPLC) system model Biologic-HR. The native and boiled ricin was dissolved in 0.005 M phosphate buffer containing 0.2 M NaCl, pH 7.2 and 1.0 mg native and boiled ricin was applied on Bio-Gel® A 0.5-m (Bio-Rad) column (30 × 1 cm) separately. The toxin was eluted with 0.005 M phosphate buffer containing 0.2 M NaCl, pH 7.2. The retention time of both native and boiled ricin was determined.

Latex Agglutination Assay

The ricin antibodies prepared in house were used for the sensitization of latex particles. Latex particles were sensitized as described elsewhere (21). The sensitized latex particles (SLP), native and boiled ricin (1 mg/mL) were placed separately in an equal volume (10 μL) on smooth surface glass plate or glass slide and mixed with wooden tooth pick and by rotating the plate manually. The test results were scored as follows: 4+, rapid agglutination of 100% of SLP, with formation of ring (within 10 sec); 3+, agglutination of >75% of SLP with some ring formation (within 1.0 min); 2+, agglutination of 50% of SLP, with no ring formation (within 2.0 min); 1+, fine particle agglutination usually involving 25% of SLP (within 3–4 min). Agglutination after 5 min should not be scored. For control, equal volume of Borate-BSA buffer (10 μL) and SLP (10 μL) was mixed as per test and used as negative control for grading purpose.

Dot ELISA

Dot ELISA was performed for the detection of ricin. Native and boiled ricin were coated on Dot ELISA comb at a concentration of 1 μg/Dot. Ricin-coated Dot ELISA comb was incubated with rabbit antiricin antibodies (1:1000 dilution) and goat anti-rabbit IgG-HRP conjugate (1:1000 dilution). The conjugate-treated strips were then developed by using chromogenic substrate solution containing DAB and 4-chloro-1-naphthol. The results were recorded visually by the presence of brown color dot.

Immuno-chromatographic Techniques (ICT)-Based Assay

Immuno-chromatographic techniques-based ricin assay of native and boiled ricin was performed on Tetracore® Ricin ICT card. For ICT assay, native and boiled ricin were dissolved in equal amounts (1.0 mg/mL) in PBS and 50 μL of sample was applied on the sample pad at the bottom of the test strip. The ricin if present in the test sample combines with the colloidal gold-conjugated antiricin antibodies in colloidal gold release pad present just below the sample pad. The combined solution then moves upward by capillary action, and the ricin bound with colloidal gold-conjugated antiricin antibodies in the solution combines with the antiricin IgM (test antibody) coated on the nitrocellulose membrane and thus forming a dark color band at the site where the test antibodies have been coated. The colloidal gold-conjugated antiricin antibodies still move further and combine with control antibody that is goat anti-rabbit IgG coated on the top end of the test strip. Thus, positive test results in the formation of two visible bands at the control as well as test antibody-coated site whereas negative test results in only one visible band at the control antibody-coated site. The test is considered invalid if no line appears on the test strip or only one line appears at the test antibody coated site. The excess of test sample solution moves on further and is absorbed by the absorbent pad present just above the nitrocellulose membrane. The assay is completed in less than 10 min.

SDS–PAGE of Native and Boiled Ricin

The native and boiled ricin samples were prepared as mentioned in preparation of ricin and animal exposure for toxicity studies section. The native and boiled ricin samples were mixed with an equal volume of SDS–PAGE sample buffer and boiled for 3 min in a boiling water bath. Approximately 10 μg/10 μL of native and boiled ricin was loaded in each well for the electrophoresis. The ricin samples were separated on SDS–PAGE using Bio-Rad electrophoretic apparatus. The SDS–PAGE was performed according to Laemmli (22).

Western Blot Analysis

Western blot analysis was performed as described by Chaponi and Migliorini (23). Native and boiled protein samples were separated on SDS–PAGE and electrophoretically transferred onto a nitrocellulose membrane filter, using an electro-blotting apparatus (Bio-Rad). After protein transfer, polyvinylidene difluoride membrane (0.20 μm, pore size; Pierce Biotechnology Inc., Rockford, IL) was blocked overnight at 4°C with 5% skimmed milk powder in PBS and incubated for 90 min with antiricin antibody at 1:2000 dilution. Treated membrane was incubated with goat anti-rabbit IgG-HRP conjugate (1:50,000) for 90 min at room temperature. The membrane was washed again and developed using an enhanced chemiluminescent detection system (ProteoQwest™ Chemiluminescent Western blotting kit; Sigma) according to manufacturer's protocol and the image was taken on Pierce CL-XPosure™ X-ray films.

Acidic Nondenaturing Gel Electrophoresis

Acidic nondenaturing gel electrophoresis was performed according to Hames (24) with slight modifications. The 5% stacking gel was made in glacial acetic acid/KOH solution (final concentration was 0.75% and 120 mM, respectively, pH 5.9). Ammonium per sulfate and N,N,N′,N′-tetramethyl ethylenediamine (TEMED) concentration were 0.7% and 0.06%, respectively. The 15% resolving gel was made in acetic acid/KOH solution (final concentration 13.25% and 30 mM, respectively, pH 2.9). The concentration of ammonium per sulfate was similar to that of stacking gel. TEMED concentration was increased to 0.6%. Under these conditions, polymerization took about 30–60 min. Electrode buffer was 0.16% acetic acid containing 0.6% β-alanine, pH 2.9. Loading buffer was 0.8% glycerol and 2% methylene blue. Samples (10 μg/10 μL) were mixed with equal volumes of loading buffer for application onto the gel. Electrophoresis was performed in the cold (4°C), at 200 V, for 75 min. After electrophoresis, the gel was rinsed with water and placed in Coomassie Blue stain (0.4% dye made in 50% methanol/10% acetic acid). Distaining was carried out in 30% methanol/10% acetic acid solution.

Results

In Vivo *Toxicity of Native and Boiled Ricin*

There are sufficient possibilities of ricin use by terrorist groups, because of its easy availability. There are differences in toxicity and molecular structures of both native and boiled ricin. The remarkable difference was observed for *in vivo* toxicity studies of native and boiled ricin. Table 1 represents toxicity comparison of native and boiled ricin at various time intervals. No mortality was observed following 25 mg/kg body weight dose in case of boiled ricin for 3.75, 7.5, 15.0, 30.0, and 60.0 min. Treated animals also didn't show any significant change in body weight for a period of 2 weeks. However, the native ricin at the doses of 2.5, 5, 10, and 20 μg/kg body weight was lethal to the animals. The native ricin treatment groups at the doses of 2.5, 5, 10, and 20 μg/kg body weight show 25%, 50%, and 100% mortality, respectively.

Chromatographic Profile of Native and Boiled Ricin

Figure 3 shows the retention time of native (Fig. 3*A*) and boiled ricin (Fig. 3*B*). The elution profile of the native ricin and boiled

TABLE 1—*Toxicity comparison of native and boiled ricin**.

Group	Dose of Ricin	Time of Boiling (min)	No. of Animals Dosed	No. of Animals Died	7-Day Body Weight (g)	14-Day Body Weight (g)
Control	Water	–	4	0	106.3 ± 3.5	115.38 ± 4.2
Ricin (native)	2.5 μg/kg	0	4	1	97.82 ± 2.6	101.91 ± 2.8
	5.0 μg/kg	0	4	2	–	–
	10.0 μg/kg	0	4	4	–	–
	20.0 μg/kg	0	4	4	–	–
Ricin (boiled)	25 mg/kg	3.75	4	0	105.2 ± 2.8	109.04 ± 3.8
	25 mg/kg	7.5	4	0	106.2 ± 3.2	108.78 ± 3.7
	25 mg/kg	15.0	4	0	100.0 ± 1.8	100.0 ± 2.9
	25 mg/kg	30.0	4	0	100.28 ± 4.4	99.78 ± 6.8
	25 mg/kg	60.0	4	0	100.0 ± 3.4	94.07 ± 7.2

*The male mice weighing between 30 to 35 g were injected native and boiled ricin through i.p. route. Animals were observed for 14 days and data were collected on mortality and body weight. $n = 4$.

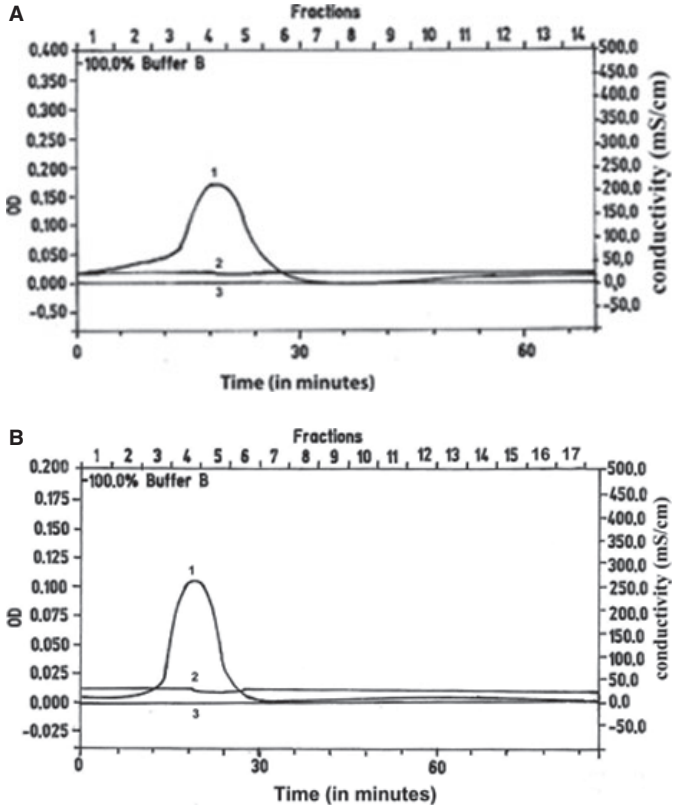

FIG. 3—*(A) Column chromatography of native ricin on Bio-Rad MPLC system model Biologic-HR. The native ricin (1.0 mg/mL) was applied on Bio-Gel® A 0.5 m (Bio-Rad) column (30 × 1 cm). Ricin was eluted with 0.005 M phosphate buffer containing 0.2 M NaCl, pH 7.2. The retention time of native ricin was determined. 1: Optical density; 2: conductivity, and 3: base line. (B) Column chromatography of boiled ricin on Bio-Rad MPLC system model Biologic-HR. The boiled ricin (1.0 mg/mL) was applied on Bio-Gel® A 0.5 m (Bio-Rad) column (30 × 1 cm). The boiled ricin was eluted with 0.005 M phosphate buffer containing 0.2 M NaCl, pH 7.2. The retention time of boiled ricin was determined. 1: Optical density; 2: conductivity, and 3: base line.*

ricin is similar confirming that the boiled ricin retains its structure and there is not much difference in its molecular weight. The retention time of both native and boiled ricin was found to be 18.01 and 18.46 min, respectively. Only 45 sec delay was observed in the retention time of boiled ricin, which is insufficient to distinguish the native and boiled ricin.

Detection of Native and Boiled Ricin by Latex Agglutination and Dot ELISA

Figure 4 represents the results of latex agglutination. Both native and boiled ricin samples at a concentration of 1 mg/mL were detected by SLP with third grading of agglutination (within 1–3 min). No difference was observed in the agglutination of native and boiled ricin. Dot ELISA was also performed with native and boiled ricin. Ricin samples were coated on Dot ELISA comb at a concentration of 1 μg/2 μL. The result of boiled ricin and native ricin indicates that there is no apparent change in the dot up to 3.75 min boiled ricin. It is very clear that the antiricin antibodies can't differentiate between native and boiled ricin. As the ricin boiling time is increased further, more protein aggregates are formed and the intensity of dot is reduced (Fig. 5).

Detection of Native and Boiled Ricin by ICT

Detection of ricin through ICT-based card is presented in Fig. 6. The ICT card test for boiled ricin and native ricin (Fig. 6*B*,*C*) shows equal intensity line for both the native as well as boiled ricin. It indicates that the antibodies coated on Tetracore® Ricin ICT card were also not able to distinguish native and boiled ricin.

Molecular and Conformational Differences of Native and Boiled Ricin

The results of nonreduced SDS–PAGE of the native and boiled ricin are shown in Fig. 7. It clearly indicates that upon boiling the

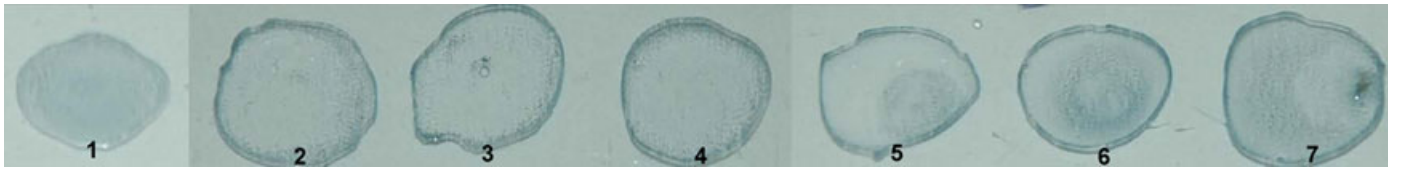

FIG. 4—*Detection of ricin by Latex agglutination test after denaturing ricin in boiling water at various time intervals. 1: Control; 2: ricin; 3: 3.75 min boiled ricin; 4: 7.5 min boiled ricin; 5: 15.0 min boiled ricin; 6: 30.0 min boiled ricin; 7: 60.0 min boiled ricin.*

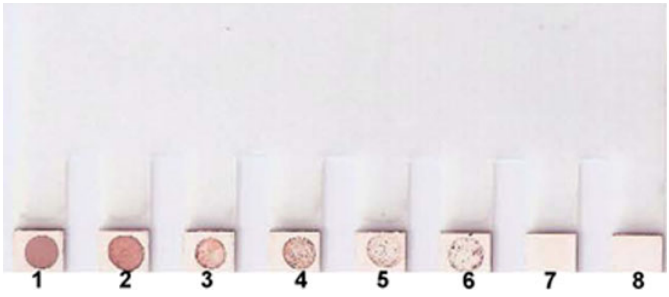

FIG. 5—*Detection of ricin by Dot-ELISA after denaturing in boiling water for various time intervals. 1: Ricin; 2: 3.75 min boiled ricin; 3: 7.5 min boiled ricin; 4: 15.0 min boiled ricin; 5: 30.0 min boiled ricin; 6: 60.0 min boiled ricin; 7 (−Ag) and 8 (−Ab): Controls.*

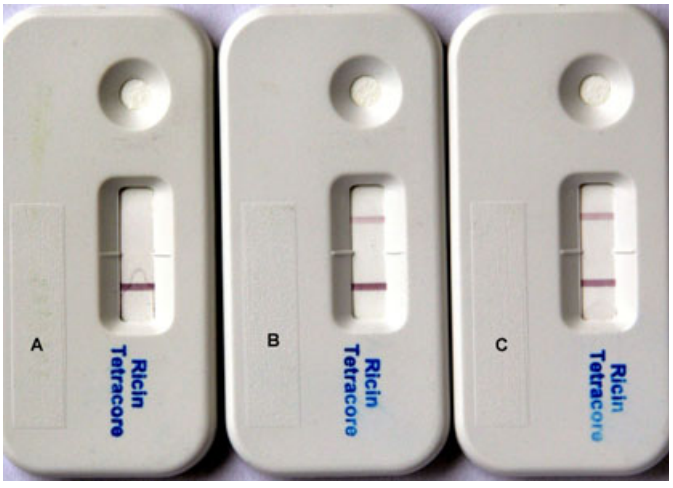

FIG. 6—*Detection of ricin on immuno-chromatographic strip after denaturing ricin in boiling water for various time intervals. A: Control; B: 15.0 min boiled; C: native ricin; ricin.*

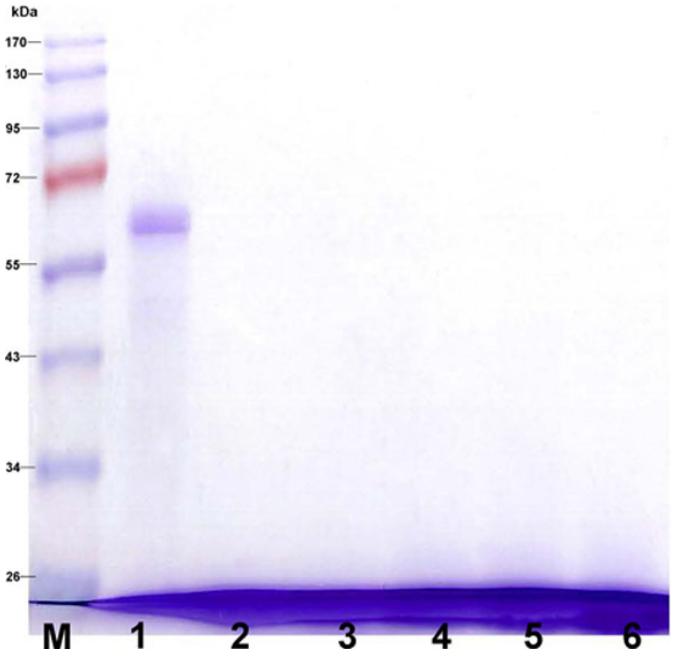

FIG. 7—*SDS–PAGE of native and boiled ricin. Lane (M): molecular weight marker; 1: native ricin; 2: 3.75 min boiled ricin; 3: 7.5 min boiled ricin; 4: 15 min boiled ricin; 5: 30 min boiled ricin; and 6: 60 min boiled ricin.*

protein molecule undergoes irreversible changes/degradation and the degraded products can be visualized at the bottom of the gel but remain unresolved. The western blot was carried out for both the samples at similar concentration. The native ricin can be detected after an exposure time of 2 min on X-ray film (Fig. 8*A*), but detection of boiled ricin for 3.75 min requires a longer exposure time of 30 min (Fig. 8*B*). The detection of ricin after boiling for 7.5, 15.0, 30.0, and 60.0 min was not possible even after prolonging the exposure time (Fig. 8*B*).

Figure 9 shows the results of native PAGE of native and boiled ricin on acidic nondenaturing gel electrophoresis. The additional evidence of denaturation/degradation of protein by boiling at 3.75, 7.5, 15.0, 30.0, and 60.0 min was obtained by acidic nondenaturing gel electrophoresis native PAGE analysis. The band intensities of

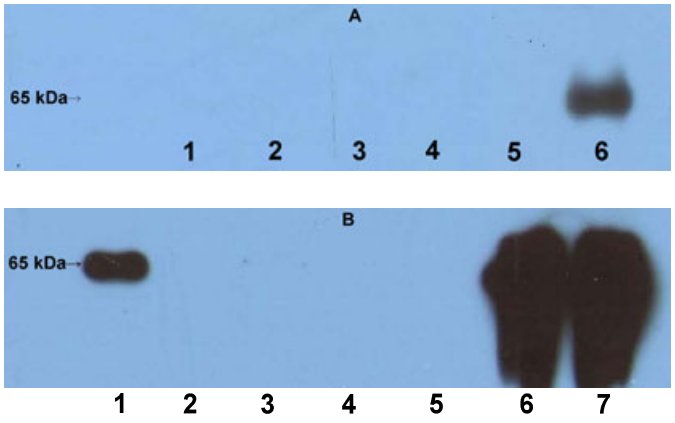

FIG. 8—*(A) Western blot of native and boiled ricin. 1–5: 3.75–60 min boiled ricin and 6: native ricin (X-ray film exposed for 2 min). (B) Western blot of native and boiled ricin. 1: 3.75 min boiled ricin; 2: 7.5 min boiled ricin; 3: 15 min boiled ricin; 4: 30 min boiled ricin; 5: 60 min boiled ricin; and 6–7: native ricin (X-ray film exposed for 30 min).*

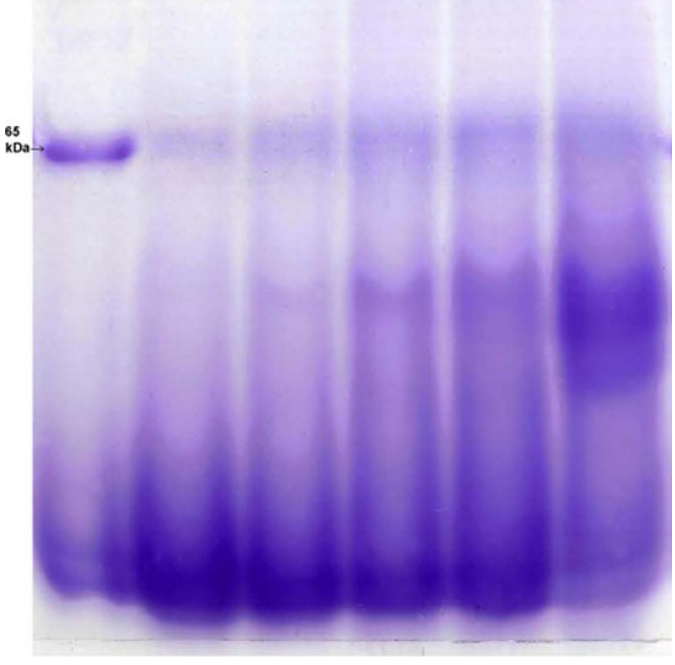

FIG. 9—*Native PAGE: Lane 1: native ricin; 2: 3.75 min boiled ricin; 3: 7.5 min boiled ricin; 4: 15 min boiled ricin; 5: 30 min boiled ricin; and 6: 60 min boiled ricin.*

native and boiled ricin samples are different but their mobility is similar. The native and boiled ricin give a ricin band, but the intensity of the ricin band in boiled samples is less in comparison with native ricin. It was also observed that as boiling time increases, the degradation of ricin is also increased.

Discussion

Ricin is a highly toxic plant protein that belongs to a group of proteins called ribosome inactivating proteins. There are many reports about extraction of ricin by individuals for criminal or terrorist activities as the purification of ricin is not very difficult. After the incident of 11/9 ricin, anthrax was seized from different locations in U.S.A., London, and other parts of the world (9,25), but most of the samples were found false. Thus, we need sensitive detection systems that can detect minute quantities of ricin or other biological warfare agents in various types of matrixes.

Ricin may vary in degree of glycosylation between different castor bean plant species as well as within the same plant as a result of multigenic expression. There are apparent differences in the primary structure of different ricin isoforms. These may result in differences in functional efficacy and toxicity of different isoforms. Various isoforms of ricin have been reported and characterized analytically as well as toxicologically (19). The term ricin applies to all the isoforms including the toxic mutant, but not to the individual side chains. In other words, ricin is characterized by the generalized structure formula A-S-S-B (Fig. 2). It means all the forms of ricin that are having A-S-S-B, whether they are active or inactive are called ricin.

According to CDC report (25), an envelope with a threatening note and a sealed container was processed at a mail processing and distribution facility in Greenville, SC, U.S.A. CDC laboratory confirmed that ricin was present in the container. CDC conducted environmental assessment and sampling at the postal facility, consisting of 70 wipe samples and five surface dust samples. All environmental samples were analyzed at CDC and were found negative for ricin. No workers had illness suggestive of ricin exposure. Statewide surveillance also did not identify any cases of ricin-associated illness. The postal facility was closed till all the environmental samples for ricin were negative (25). The havoc created by terrorists could have been addressed if the CDC possessed a system that could distinguish both native (toxic) and boiled (nontoxic or denatured) ricin.

Different assay methods have been described for the detection of ricin in solution by various authors including radioimmunoassay (11) that detected ricin in picogram level in the blood. Poli et al. (26) reported chemiluminescence ELISA that can detect ricin from diluted serum and urine samples. A sandwich ELISA, colloidal gold-based ICT, and hydrogel-based protein microchips are also reported (11,14,16). Cook et al. (27) described an antigen capture sandwich ELISA, by which they have done retrospective identification of ricin in animal's tissues following various routes of exposure. Malcolm and Brian (28) detect ricin from diluted human plasma after extraction of ricin in extraction buffer containing lactose. But not a single method is able to distinguish native and denatured ricin.

As we discussed, our main objective of this work was to find out the effectiveness of immunoassays to distinguish the native and boiled ricin. Although there are differences in toxicity and molecular characterization of both native and boiled ricin, there is a need of highly specific detection assay for their differentiation. The remarkable difference was observed for *in vivo* toxicity studies of native and boiled ricin. The denatured ricin samples at the dose of 25 mg/kg body weight were found to be nontoxic as no mortality was observed in the treated animals. Treated animals also didn't show any significant change in body weight for a period of 2 weeks. However, the native ricin at the doses of 2.5, 5.0, 10.0, and 20.0 μg/kg body weight was lethal to the animals. The mortality was observed on the third day of posttreatment. The elution profile of native ricin and boiled ricin is very similar confirming that the boiled ricin retains its structure, and there is not much difference in its molecular weight. The retention time of both native and boiled ricin was 18.01 and 18.46 min, respectively. The 45 sec delay in retention time is insufficient to distinguish native and boiled ricin. The difference in the toxicity of native and boiled ricin may be because of the conformational changes that occur during boiling.

In the present study, all the performed immunological tests viz. latex agglutination, Dot ELISA and ICT were able to detect native and boiled ricin. Hence, performed immunological methods were not able to distinguish between native and boiled ricin. SDS–PAGE of native ricin appears in 60-kDa region; however, degradation of ricin on boiling was observed. Ricin boiled for 3.75 min gave a strong blot when X-ray film was exposed for 30 min, which indicates very weak signals from ricin.

Acidic native PAGE of native and boiled ricin gave evidence that ricin is degraded on boiling. 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. Differences between RBC-Phytohemagglutinin-I, RBC-Phytohemagglutinin-II, and ricin was confirmed by acidic native PAGE at pH 2.9 (29). We concluded from this study that all the immunological methods were not able to distinguish native and denatured ricin. However, biochemical characterization using PAGE, western blotting, and toxicity evaluation gave some clue about the nature of toxin. We require a highly sensitive and specific test system which can distinguish between toxic and nontoxic form of toxins, to help overcome man-made/terrorist-created havocs in future. In this regard, advanced analytical techniques using MALDI-TOF/MS, LC-ESI/MS, and MS-MS may recognize some ricin specific peptides, and may distinguish native/boiled ricin. The present study will generate interest in the scientific community to find out some specific measures to fight against terrorism in future.

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