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Differential toxicity profile of ricin isoforms correlates with their glycosylation levels

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ABSTRACT

Ricin is one of the most potent and deadly plant toxins from the seeds of *Ricinus communis*. In view of its high toxicity, ricin is being used as an immunotoxin in cancer therapy. Ricin also has several isoforms with differential glycosylation depending on the seed variety. Our study shows three isoforms designated 1, 2 and 3, which differed in their surface charge, resulting in a different behavior on cation exchange chromatography, two dimensional (p*I* 5.5–8.7) and native PAGE. The molecular masses of isoform-1, 2 and 3 were measured as 63.55 kDa, 64.03 kDa and 62.8 kDa, respectively, by MALDI-TOF/MS. *In vitro* studies with monkey kidney (Vero) cells showed a time dependent increase in cytotoxicity of the isoforms evaluated by extracellular lactate dehydrogenase activity and mitochondrial dehydrogenase assay. These isoforms also induce oxidative stress and DNA damage. Among the isoforms, isoform-3 was quick to generate reactive oxygen species (ROS), (in 90 min) and exhibited maximum cytotoxicity. Morphological changes, catalase activity and DNA fragmentation were significantly higher with isoform-3 treatment compared to others. The glycosylation studies by MALDI-TOF/MS showed that isoform-3 is highly glycosylated with high sugar levels containing more of hybrid/complex type glycopeptides with mannose as hexose units. These experimental evidences clearly suggest that isoform-3 is superior in its early ROS generation, potency to induce oxidative stress and cytotoxicity, that could be due to it's higher glycosylation levels which make isoform-3 as an ideal candidate for immunotoxin studies.

1. Introduction

Ricin is a toxic glycoprotein purified from the castor oil seeds (*Ricinus communis*). Castor bean plant is cultivated as an oil seed crop and also grown as an ornamental plant in many countries of Asia. Worldwide, one million tons of castor beans are processed annually in the production of castor oil; the waste mash from this process contains ricin. Ricin belongs to the family of type II ribosome inactivating proteins (type II RIP) which consists of two non-identical polypeptide chains linked by a single disulfide bond. Ricin is a potent inhibitor of cellular protein synthesis. There are several isoforms of ricin including ricin D, ricin E and the closely related lectin *R. communis* agglutinin (RCA). Together they account for more than 5% of the total protein present in mature seeds. Independent of isoform, ricin is a 64 kDa heterodimeric protein consisting of a 32 kDa A chain (RTA), which kills the cell due to its enzymatic activity, and a galactose-binding ricin B-chain (RTB), which is responsible for cell binding and internalization of toxin across the cell membrane (Lord et al., 1994). The A chain is a specific RNA N-glycosidase that removes an adenine (A4324 position in rat liver DNA) residue from an exposed loop of 28S ribosomal RNA. The depurinated loop restricts the protein synthesis at the translocation step as the binding of ribosomes to elongation factors is disrupted (Irvin, 1995).

Ricin is known to exists in different isoforms which differ according to the type of seeds and variety of plant from which they are purified. There are apparent differences in the primary structure of different isoforms. These may result in differences in functional efficacy and toxicity of different isoforms. Various isoforms of ricin have been reported and characterized analytically and toxicologically (Helmy and Pieroni, 2000). It has been reported that there are two toxic ricins: ricin D and ricin E (Lin and Li, 1980). The D form is found in large grain seeds, whereas the small grain seeds contain both D and E forms of ricin toxins (Despeyroux et al., 2000). In contrast to ricin, RCA is a tetrameric protein with stronger agglutinating properties and is weakly toxic. The presence of different

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isoforms of ricin D in small and large *R. communis* beans in India has been earlier reported (Hegde and Podder, 1992).

Ricin is a potential candidate for cancer chemotherapy in the form of immunotoxins (Schnell et al., 1998) due to its extreme toxicity. It is therefore important that information on cytotoxicity of ricin isoforms should be taken into account. The cytotoxicity of ricin is mainly attributed to the inhibition of protein synthesis. However, studies have demonstrated that following administration of ricin to animals or exposure of cellular systems to ricin there are biochemical, cellular and functional disturbances consistent with the occurrence of oxidative stress (Suntres et al., 2005). This shows oxidative stress as a possible contributing mechanism in ricin induced cytotoxicity. Oxidative stress can be caused by an elevation in the steady state concentration of reactive oxygen species (ROS). Exposure of human cervical cancer cells to ricin resulted in time-dependent increases in intracellular ROS with concomitant reductions in cellular GSH levels and cell viability (Rao et al., 2005).

Ricin shows structural heterogeneity mainly due to the presence of many glycoforms (Despeyroux et al., 2000). It contains four potential N-glycosylation sites at asparagines 10 and 236 of the A-chain and asparagines 95 and 135 of the B-chain (Rutenber and Robertus, 1991). Variations in the oligosaccharide chain structure present on glycoproteins can significantly affect many protein properties such as solubility, stability, specific activity, antigenicity, resistance to protease attack and thermal denaturation (Goochee et al., 1991). There are no reports as to how this glycosylation influences the stability and toxicity of ricin and its isoforms.

Keeping the above facts in consideration, the present study is carried out to determine whether the glycosylation levels in ricin isoforms have any effect on their ability to generate ROS and toxicity.

2. Materials and methods

2.1. Chemicals

The castor seeds were purchased from the local market. 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), thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Chemicals Co. (St. Louis, MO). O-phthaldialdehyde (OPT), Hoechst 33342 were from Acros (Beigium). SOD and Catalse kits were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA) unless otherwise mentioned.

2.2. Purification of ricin

Ricin was isolated from *R. communis* seeds as described previously (Kumar et al., 2003), applying lactamyl sepharose affinity (Hegde et al., 1991) and gel filtration chromatography.

2.3. Purification of ricin isoforms

Ricin isoforms were isolated by cation-exchange chromatography as described earlier (Hegde and Podder, 1992) with some modifications. Chromatographic purification was achieved using protein purification system, Bio-Rad, USA (Biologic-HR model). The purified ricin was loaded on a CM–sepharose fast flow column (2.5 cm × 10 cm) pre-equilibrated with 10 mM sodium phosphate buffer, pH 6.5 containing 20 mM lactose. The bound proteins were eluted using an ionic gradient of 0.0–0.1 M NaCl in the buffer described above and 1 M NaCl applying the following gradient: 0–0.1 M for 2.5 h and 1 M NaCl for 0.5 h. Three fractions containing ricin were isolated and absorbance was recorded at 280 nm. The proteins under each peak were pooled separately, dialyzed against water, lyophilized and stored at −20 °C.

2.4. SDS-PAGE and acidic native PAGE

Two electrophoretic systems were used for protein characteristics: (1) SDS-PAGE in 12% gel in Laemmli buffer system (Laemmli, 1970) and (2) Acidic native PAGE in β-alanine-acetate buffer, pH 4.3. Samples were diluted (1:1) with sample buffer (50 mM Tris pH 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol and 0.04% bromophenol blue) and were boiled for 5 min. Approximately 10 μg of samples were loaded in each well for the electrophoresis. SDS-PAGE under reduced and non-reduced conditions was performed to assess the purity of ricin and its isoforms. Separation was done using Bio-Rad electrophoretic apparatus.

Acidic native PAGE was performed according to Hames (1990) with slight modifications. The 5% stacking gel was prepared in 120 mM KOH, 0.75% acetic acid, pH 5.9. The ammonium persulfate and TEMED concentration was 0.7% and 0.06%, respectively. 15% resolving gel was prepared in 30 mM KOH, 13.25% acetic acid, pH 2.9. The concentration of ammonium persulfate 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, 125 mM KOH and 0.75% acetic acid (pH 5.9). Samples (10 μg) 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. 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.

2.5. Two-dimensional gel electrophoresis (2-DE)

2-DE analysis was carried out according to O'Farrel (1975). For first dimension 300 μg of protein in 130 μl 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 Vh 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 and their images were captured.

2.6. Molecular weights measurement and protein identification by mass spectrometry

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, 337 nm) 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.

For routine MALDI analysis of purified protein, dried droplet method was used. The protein sample (1 μg) 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.

For in-gel trypsin digestion, the protein spots were manually excised from 2-DE gel and washed thrice with 400 μl 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 200 μl of 100% ACN and dried in speed vac centrifuge. The protein contained in the gel was subjected to digestion using 25 μl (20 μg/100 μl) 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 200 μl 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).

2.7. Cell culture and treatment

Vero cell line (monkey kidney cells) was obtained from NCCS, Pune. The cells were grown in Eagle's Minimum Essential Medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 1.5 g/l sodium bicarbonate and gentamycin (80 μg/ml). The cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. Vero cells grown in either 24-well tissue culture plates or 25 mm^2 tissue culture flasks. The cells were treated with 50% inhibitory concentration (IC_{50}) of ricin and its isoforms for time course experiments on cell viability by LDH leakage and MTT assay. For various biochemical endpoints and DNA fragmentation assay treatment duration was 24 h at IC_{50} dose. Morphological changes of Vero cells were photographed with an inverted phase contrast microscope (Olympus IX51) at 24 h time point with or without ricin isoform exposure.