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SHORT COMMUNICATION

Purification and Biochemical Characterisation of Ricin from Castor Seeds

Om Kumar, A. B. Nashikkar, R. Jayaraj, and R. Vijayaraghavan
Defence Research & Development Establishment, Gwalior-474 002

and

A.O.Prakash
Jiwaji University, Gwalior-414 Oil

ABSTRACT

Ricin is a highly toxic plant toxin of *Ricinus comtnunis* seeds, commonly known as castor seeds. The toxin was extracted and purified using affinity and size exclusion chromatography. The purity of ricin was evaluated by the sodium dodecylsulphate-polyacrylamide gel electrophoresis. Purified ricin gives a single band under non-reduced condition and two bands under reduced condition. The molecular weight of ricin was 65,0000 approx. The subunit structure of ricin on treatment with p-mercaptoethanol (1 %) at molecular level revealed that the reducing agent converts ricin into two peptides. The molecular weight of these two peptides was estimated to be 34000 and 32000. The western-blot analysis revealed two dots for its two peptides in 29 kDa to 36 kDa regions. The heamagglutination litres for ricin and *Ricinus communis* agglutinins were 1:8 and 1:256. The purity of purified ricin was further confirmed by the electrophoresis and the western-blot analysis. The Indian variety of castor seeds, known as *Ricinus communis* used in this study, contains approx. 0.12 per cent ricin.

Key words: Ricin, castor seeds, purification, gel filtration, polyacrylamide gel electrophoresis, western-blot analysis, blotting, heamagglutination, SDS-PAGE, sodium dodecylsulphate-polycrylamide gel electrophoresis, *Ricinus communis*

1. INTRODUCTION

Ricin is a proteinaceous toxin[1] contained in the seeds of different varieties *of Ricinus communis* plant, commonly known as castor plant. Ricin is a small dipeptide molecule (mol wt approx. 65 kDa) containing A chain (-32 kDa) and B chain (~32-34 kDa) linked with a disulphide bond[2]. Ricin A chain inactivates 60S ribosomal subunit by disrupting the binding site for elongation factor (EF-2), and thus prevents the formation of the initiation complex by cleaving a specific adenine residue at 4324 position in mammalian 28S rRNA and inhibits protein synthesis, leading to cell death[3,4]. Ricin may vary in degree of glycosylation between the 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

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been reported and characterised analytically and lexicologically[5].

According to the Organisation for Prohibition of Chemical Weapons (OPCW)[6] report, ricin was assessed as a candidate of chemical warfare agent (CWA) during world war I. During world war II, ricin was only field tested and not actually used. Aerosol dissemination tests demonstrated that ricin could be used as an effective chemical weapon. Despite its heat sensitivity, the toxin survived dissemination by explosives and retained much of its toxicity. Ricin is considered as a potent chemical warfare agent[6]. In spite of the Chemical Warfare Convention (CWC), ricin can be misused by any country due to the wild growth *ofRicinus communis* plant. In contrast to global scenario not much work has been done in India on ricin, although, India is the large producer of castor seeds. In the present study, ricin was extracted and purified from Indian castor seeds, and characterised biochemically for its purity, and subunit structure at the molecular level.

2. MATERIALS & METHODS

2.1 Chemicals

Bio-gel (A-0.5m gel) was obtained from the Bio-Rad, USA. Sepharose 4B, molecular weight markers, anti-ricin antibody, 3,3'-diaminobenzidin (DAB) and all electrophoresis chemicals were purchased from the Sigma Chemicals Co (St. Louis, MO). The castor seeds were purchased from the local market. Other chemicals used were purchased from the SRL/Himedia/Merck (India).

2.2 Extraction & Purification

Decorticated castor seeds were defatted by grinding in equal volumes of ether and centrifuged at 3000 g for 10 min. The supernatant was discarded and the pellet was resuspended and again grounded in equal volume of ether. This procedure was repeated four times. The final pellet was collected and air dried[2].

Decorticated and defatted castor meal was soaked in five volume of distilled water and the *pH* was adjusted to 4.0 by adding dilute acetic acid. The suspension was homogenised at a maximum speed in a grinder mixture at 4 °C in ten sequences of 1 min each with 30 min interval and left overnight. The homogenate was then centrifuged at 8000 g for 10 min. Supernatant was collected, adjusted to 60 per cent ammonium sulphate saturation, left overnight at 4°C and centrifuged at 10,000 g for 30 min in a refrigerated centrifuge. Pellet was collected and dissolved in 0.005 M sodium phosphate - 0.2 M *NaCl* buffer *pH* 7.2 phosphate buffer saline (PBS), and exhaustively dialysed against PBS till complete removal of ammonium sulphate. The dissolved pellet was concentrated by lyophilisation and referred as crude ricin[7].

The affinity and gel filtration purification of ricin was carried out according to Griffiths[8]. The crude ricin was loaded on affinity column on acid-treated sepharose-4B matrix (0.1 M *HCl*, 3 h at 50 °C) in *NaCl* solution (0.5 M). The column was washed with PBS for several hours (till the optical density reached below 0.05) to remove the unbound proteins. The matrix-bound lectins were eluted with (0.5 M) *NaCl* solution containing (3-D-galactose (0.1 M). Fractions were collected manually, and absorbance was recorded at 280 nm. Protein containing fractions were pooled and concentrated. These were then separated on the basis of their size difference, using bio-gel (A-0.5m gel) chromatography.

2.3 Gel Electrophoresis & Molecular Weight Determination

The purified preparations were assessed for .their purity, using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reduced and reduced conditions according to Laemmli[9]. The molecular weight was determined according to Weber and Osborn[10].

2.4 Western-blot Analysis

The western-blot analysis was performed as described by Sominskaya". After protein transfer, nitrocellulose sheet (0.20 mm, pore size; Schleicher & Schuell) was blocked at room temperature for 60 min with skimmed milk powder (5 %), in PBS and incubated for 90 min with anti-ricin antibody

at 1:1000 dilution. Treated membrane was incubated with goat anti-rabbit IgG-HRP conjugate (1:2500) for 90 min at room temperature. The nitrocellulose sheet was washed three times for 5 min with phosphate buffer saline-Tween 20 (PBST) and developed with 3,3'-diaminobenzidine.

2.5 **Heamagglutination Assay**

Heamagglutination assay was performed in microtitre plates (Nalge Nunc International, Denmark). Each well contained 100 ml of diluent (0.05 M *Tns-HCl/O.l* M *NaCl* buffer, *pH* 7.6), 100 ml lectin (0.1 M-g/ml) in the same buffer and was serially diluted. One per cent suspension of sheep red blood cells (50 nl) in *NaCl* (0.15 M) was added to each well and gently mixed. The plates were incubated at room temperature for 3 h and the last well showing heamagglutination was taken as the end point. The heamagglutination titre was expressed as the reciprocal of dilution factor.

3. RESULTS & DISCUSSION

Ricin is a protein component *of Ricinus communis* seeds which is exquisitely toxic to mammalian cells. Stillmark first isolated this toxic protein[12]. The crude ricin obtained after extraction and dialysis was first purified by the affinity chromatography on acid-treated sepharose 4B (Fig.l). Further, purification of affinity-purified lectins using gel filtration chromatography is presented in the Fig. 2. Separation takes place on the basis of molecular size. Ricinus agglutinin elutes first and ricin elutes later. The Indian variety of castor seeds used in this study contains ricin (0.12 %), approximately of whole castor seeds. Olsnes[13] described various methods for extraction, isolation, and purification of abrin and ricin from *Abrus precatorius* and *Ricinus communis* seeds, respectively. The toxin ricin was purified by the CM-cellulose chromatography, followed by the affinity chromatography on sepharose 4B. Various

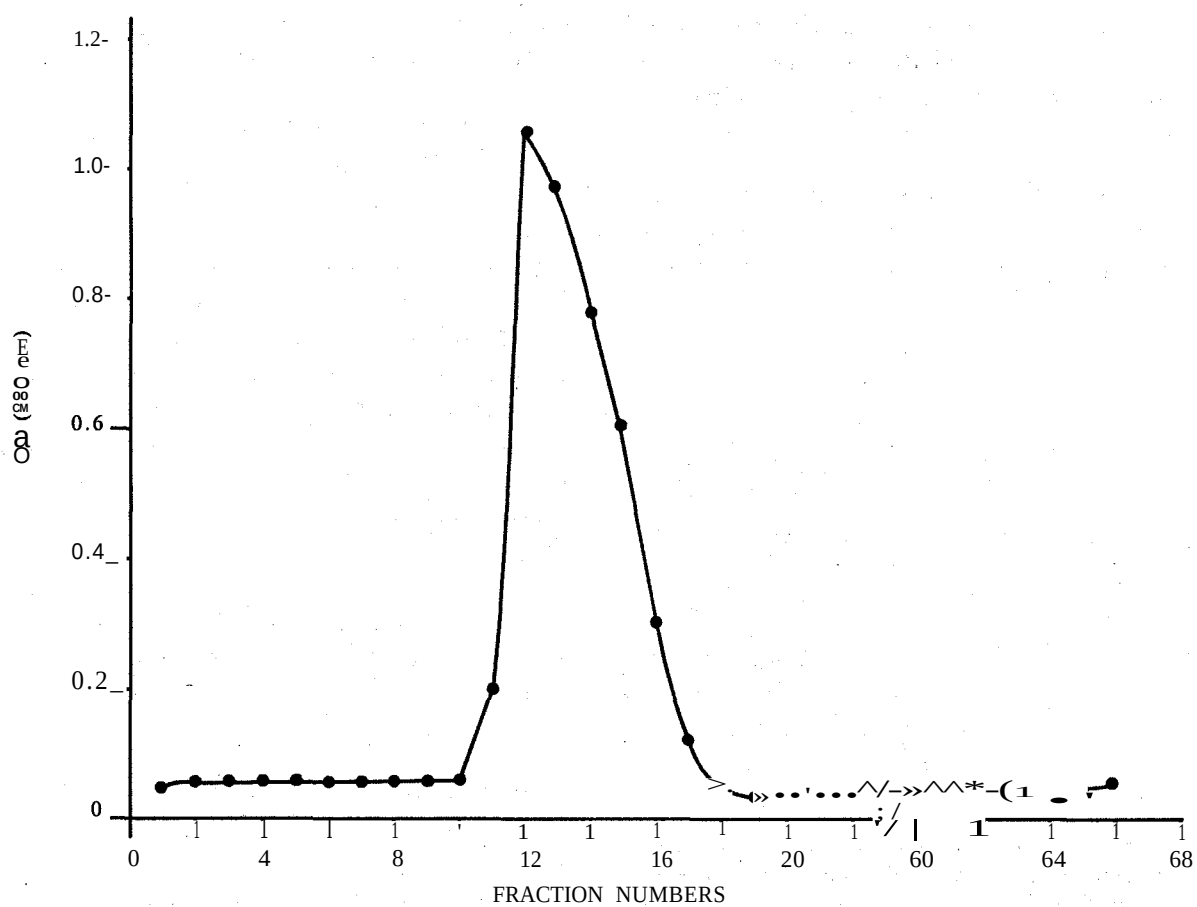

Figure 1. Affinity chromatography on acid-treated sepharose 4B of ammonium sulphate fractionated *Ricinus communis* agglutinins

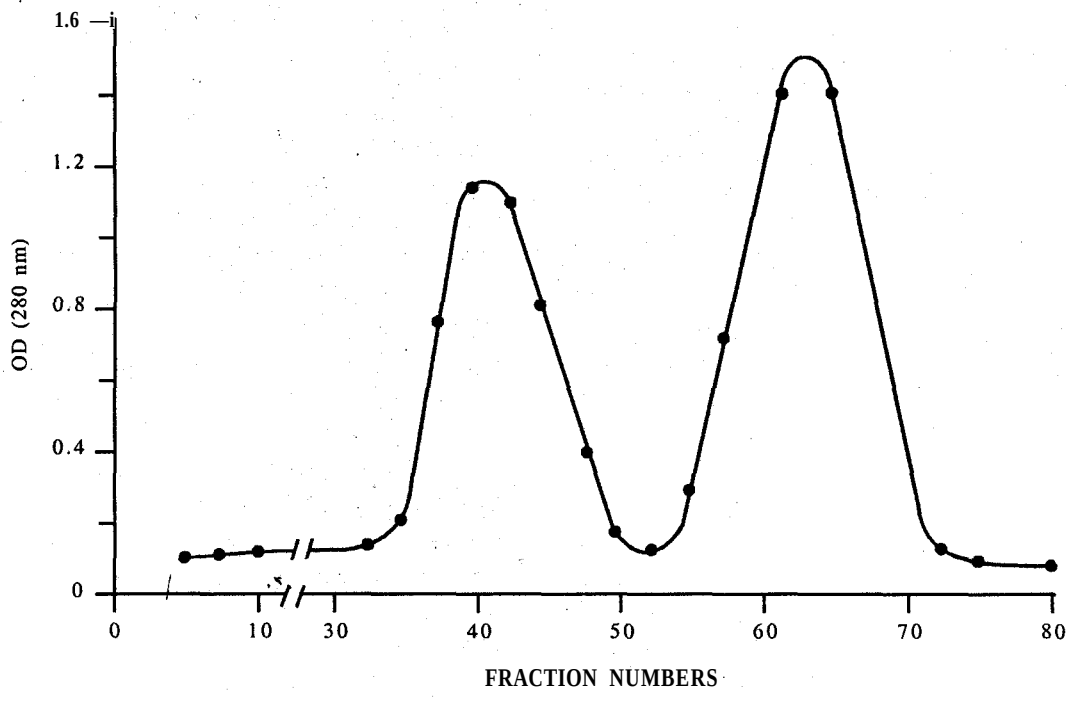

Figure 2. Size-exclusion chromatography of affinity-purified agglutinins

chromatography techniques using different matrices have been used for the purification of ricin[14]. A single-step method for the purification of ricin toxin from castor seeds is also reported[15].

The purity of lectins was evaluated by SDS-PAGE technique. The relative migration rate of ricin under reduced and non-reduced conditions to those of known molecular weight markers have been presented in the Fig. 3.

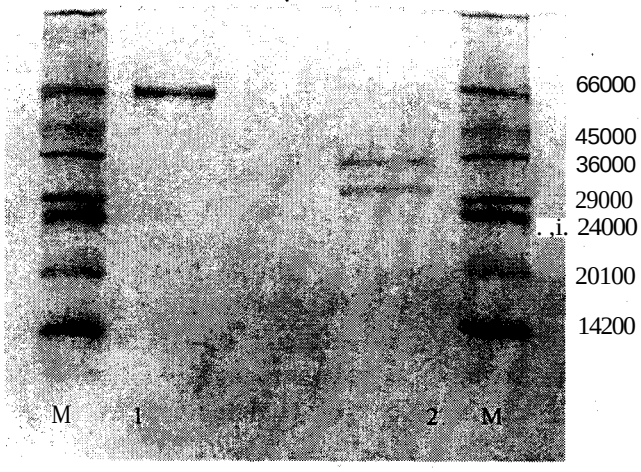

Figure 3. SDS-PAGE of purified ricin on 10 per cent acrylamide gel. M the molecular weight markers; (1) in the absence of (3-mercaptoethanol, and (2) in the presence of P-mercaptoethanol.

Under non-reduced condition, ricin gives single band. When ricin is treated with P-mercaptoethanol (1%), it gives two bands which represent A and B subunits of the ricin. No other band has been observed. The molecular weight of the ricin has been found to be 65,000 approx. The molecular weight of its two peptides was estimated to be 34,000 kDa and 32,000 kDa. Variations have been reported in the literature regarding molecular weight of ricin and its subunits. Ricin having molecular weight from 60,000 kDa to 65,000 kDa by SDS-PAGE technique has been reported[16]. The ricin subunits, having molecular weight 29,500 kDa and 34,000 kDa after P-mercaptoethanol treatment have been described[17]. Under reducing condition, the ricin appeared to have two subunits, corresponding to the molecular masses of 30 kDa and 32 kDa[17].

Figure 4 shows the transblot of SDS-PAGE of ricin toxin in various concentrations, starting from 2 ng to 10 (Xg per well. The western-blot analysis revealed two dots for its two peptides in 29 kDa to 35 kDa regions. The blot was prominent even at 2 (Ag per well. The blot does not show any other

Figure 4. Western-blot analysis of ricin: lane (1) = 2 (j,g; lane (2) = 5 jig, and lane (3) = 10 ug.

band even at high concentration of protein (10 (Xg/well) which further confirms the purity of purified toxin.

The heamagglutination assay titre for ricin and *Ricinus communis* agglutinins were found to be 1:8 and 1:256 (data not shown). The low heamagglutination assay activity further confirms the purity of purified ricin. Ricinus agglutinin (RCA-I) is known for its high agglutination activity and low toxicity, while ricin is having high toxicity and low agglutination activity. In the present study, heamagglutination assay for ricin and ricin agglutinin were carried out and heamagglutination assay activity was similar as already reported[14]. Ricin was purified from Indian castor seeds and purity of toxin was confirmed electrophoretically. The molecular weight of ricin and its two subunits were found to be 65,000 kDa, 34,000 kDa, and 32,000 kDa, respectively. The ricin content of castor seeds used in this study was 0.12 per cent approx.

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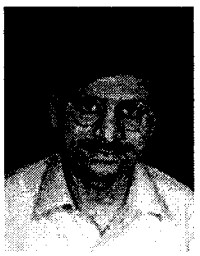
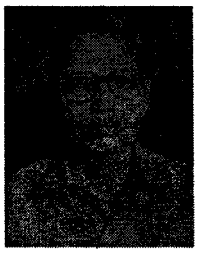
Contributors



Dr Om Kumar obtained his MSc (Biochemistry) from the I.V.R.I, Izatnagar and PhD from the Jiwaji University, Gwalior. He joined DRDO at the Defence Research Laboratory (DRL), Tezpur, as Scientist B in 1989. At present, he is working as Scientist E at the Defence Research & Development Establishment (DRDE), Gwalior. His areas of interest include: Evaluation of toxicity of chemical warfare agents, development of detection system for plant toxins and screening their antidotes. He has published 22 research papers in national/international journals.



Mr A.B. Nashikkar obtained his MSc (Chemistry) from the Jiwaji University, Gwalior. He joined DRDE, Gwalior, in 1975 and since then he is working on biochemical toxicology. He has published several research papers in national/ international journals.