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ISOLATION AND PARTIAL CHARACTERISATION OF HIGHLY TOXIC LECTINS FROM *ABRUS PULCHELLUS* SEEDS

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M. V. Ramos, D. M. Mota, C. R. Teixeira, B. S. Cavada and R. A. Moreira. Isolation and partial characterisation of highly toxic lectins from *Abrus pulchellus* seeds. *Toxicon* **36,** 477–484, 1998.—The seeds of *Abrus pulchellus*, sub-specie *tenuiflorus*, belonging to the *Leguminosae*, subfamily *Papilionoideae* contain highly toxic lectins exhibiting specificity for galactose and galactose-containing structures. The toxins which agglutinate rabbit erythrocytes, present a highly toxic activity *in vivo* when injected in the peritoneal cavity of mice ($LD_{50} = 31\ \mu g \cdot kg^{-1}$) or when tested with the microcrustacean *Arthemia salina* ($LD_{50} = 3.5\ \mu g \cdot ml^{-1}$). The active fraction was purified in a single step, by affinity chromatography on a Sepharose-4B column. The purified toxins migrated as two single bands of Mr 63 000 and 61 500 Da (SDS-PAGE) and Mr 31 500 and 29 000 Da (SDS-PAGE with 2-mercaptoethanol), respectively, suggesting the presence of disulphide-bridge interchains as occurs in other plant toxins. The antibodies anti-*A. pulchellus* toxins did not recognize ricin preparation and only partial identity was observed to *A. precatorius* toxic lectins prepared in a similar way to ricin and *A. pulchellus* toxins. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The detection of highly toxic lectins in tissues and organs of unrelated plants have been largely reported (Olsnes *et al.*, 1982; Vasconcelos *et al.*, 1994; Girbés *et al.*, 1996). They were found specially in roots (Olsnes *et al.*, 1978), seeds (Wei *et al.*, 1974), leaves (Lee *et al.*, 1992) and also in different organs in the same plant (Stirpe *et al.*, 1985). In some species, non-toxic lectins occur jointly with non-agglutinating but toxic lectins (Olsnes *et al.*, 1974a,b; Wei *et al.*, 1974). The main characteristic of the lethal-toxic lectins is their highly specific and potent enzymatic activity on eukaryotic 28S ribosomal RNA subunit, blocking the protein synthesis and leading to cell death (Endo and Kunio, 1987;

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Endo *et al.*, 1991). Recently, the best studied plant toxins; abrin, ricin and mistletoe, have been used in the construction of chimerical structures called immunotoxins, applied in chemotherapy and as a tool to study protein interaction and translation through biological membranes (Endo *et al.*, 1991; Wawrzynczak *et al.*, 1991). Despite the fact that toxic lectins, as that above, possess a large number of very interesting molecular and functional properties and that they have been first detected in more than a century, they remain under intensive investigation (Bushueva and Tonevitsky, 1988; Walles *et al.*, 1991; Citores *et al.*, 1993; Ferreras *et al.*, 1993). Moreover the potential pharmacological application of these proteins have stimulated the continuous investigation on isolation and characterisation of new lectins with highly toxic properties. We report the isolation of the galactose-binding lectins from *Abrus pulchellus* seeds which seems to produce similar effects to that from type II RIPs, proteins composed of a lectin chain (haptomer) and of an *N*-glycosidase enzymatic chain (efetomer) which are highly toxic to eukaryotic ribosomes.

EXPERIMENTAL PROCEDURES

General

Seeds of *Abrus pulchellus* var. *tenuiflorus* were collected in Ceará, Brazil. The plant material was examined and botanical identification was carried out by the Department of Biology, Federal University of Ceará. Sepharose-4B and Superose 12HR were obtained from Pharmacia Fine Chemical (Sweden). The carbohydrates, molecular weight markers and electrophoresis reagents were purchased from Sigma Chemical Company (U.S.A.). Other chemicals were of analytical grade.

Crude extract preparation

Dehulled seeds of *A. pulchellus* were ground in a mixer and the fine flour obtained was suspended (1:10 w/v) in the extraction buffer (0.1 M Tris–HCl pH 7.6, containing 0.15 M NaCl) for 2 h and centrifuged at 15 000 *g* at 4°C. The supernatant was exhaustively dialysed in distilled water and freeze-dried.

Affinity chromatography

The freeze-dried material was dissolved in the same buffer extraction (25 $mg \cdot ml^{-1}$) and loaded on a Sepharose-4B column, previously equilibrated with the same buffer. Unbound material was eluted from the column with the extraction buffer whereas the adsorbed protein containing the highest toxic and haemagglutinating activities was obtained in a single peak after elution with extraction buffer containing 0.15 M NaCl and 0.1 M galactose or alternatively with 0.1 M glycine–HCl buffer (pH 2.6) containing 0.15 M NaCl. The fractions were pooled, dialysed and freeze-dried. Unbound proteins showed a low toxicity and no haemagglutinating activity, while the retained material exhibited both activities.

Purity of proteins

The homogeneity of the purified proteins was judged by SDS-PAGE essentially as previously described (Laemmli, 1970). The samples were prepared in 0.0625 M Tris–HCl buffer (pH 6.8), with addition or not of 2-mercaptoethanol at 100°C for 10 min

and briefly centrifuged before applying to the gel. The sample corresponding to the main fractions of the purification step were compared.

Haemagglutination and haemagglutination inhibition assays

Haemagglutination assays were performed by standard methods (Moreira and Perrone, 1977) using native rabbit erythrocytes (2%). For the haemagglutinating-inhibition studies, aliquots of sugar were serially diluted in the extraction buffer, mixed with an equal volume of lectin solution containing 4 haemagglutinating units (HU) and allowed to interact for 30 min, 37°C and 30 min at room temperature. Rabbit erythrocyte suspension (2%) was added to each well and the plates were again put in stove for 30 min, 37°C. Thereafter the plates were left at 25°C for 30 min before examination. The titres of haemagglutination and inhibition of the haemagglutination were determined by macroscopic observation and the minimum concentration of sugar showing inhibition of haemagglutination was recorded.

Immunochemistry

Immunochemical studies were performed in order to establish the relationships between *Abrus pulchellus* toxins and *Abrus precatorius* and *Ricinus communis* toxic lectins isolated by a similar procedure. Antibodies against *A. pulchellus* toxic lectins (PIII) were prepared in rabbit essentially as described by Moreira and Oliveira (1983) using sublethal doses, with a first booster administrated 15 days after the initial dose. Immunodiffusion tests were performed on 1.5% agarose gels prepared with 0.9% saline containing 0.1 M galactose and 0.02% sodium azide (Clausen, 1969).

Toxic activity evaluation

The toxic activity of the lectins was determined by simple intraperitoneal injection in mice using all fractions obtained during the purification procedure. After injection of each fraction ($250\ \mu l \cdot 10^{-1}$ g of animal mass body), the toxic effects were allowed to evolve for 48 h, when the final evaluation was recorded. The method described by Litchfield and Wilcoxon (1949) was used to determine the lethal dose capable of killing 50% of the tested animals. Originally, groups of six animals and different doses of the toxins (PIII) were prepared. Each group represented a dose and each animal in the same group received the same proportion toxin/body mass. The tests were carried out during 48 h until final evaluation. The control was submitted to a physiological solution. The toxic effects of the isolated toxic lectins (PIII) were also evaluated using a microcrustacean as model, following the procedure by Vasconcelos *et al.* (1991). Viable eggs from brine shrimp (*Artemia salina*) were allowed outbreak during 24 h in sea water, when different groups of 10 animals, in triplicate, were prepared for the test. Different volumes of a lectin (PIII) solution were added to the flasks to a final volume of 5 ml corresponding to the desired concentrations. This assay was conducted twice with three replicates for each sample concentration and the results were obtained after 24 h.

RESULTS AND DISCUSSION

A crude extract was submitted to affinity chromatography on Sepharose 4B column. Two peaks of unbound material (PI + PII) were recovered and pooled together. These

fractions showed no haemagglutinating activity but were able to kill mice. The proteins retained on the column were eluted as a single peak (Fig. 1). This fraction exhibited haemagglutinating and toxic activities. The presence of toxic activity in the unbound

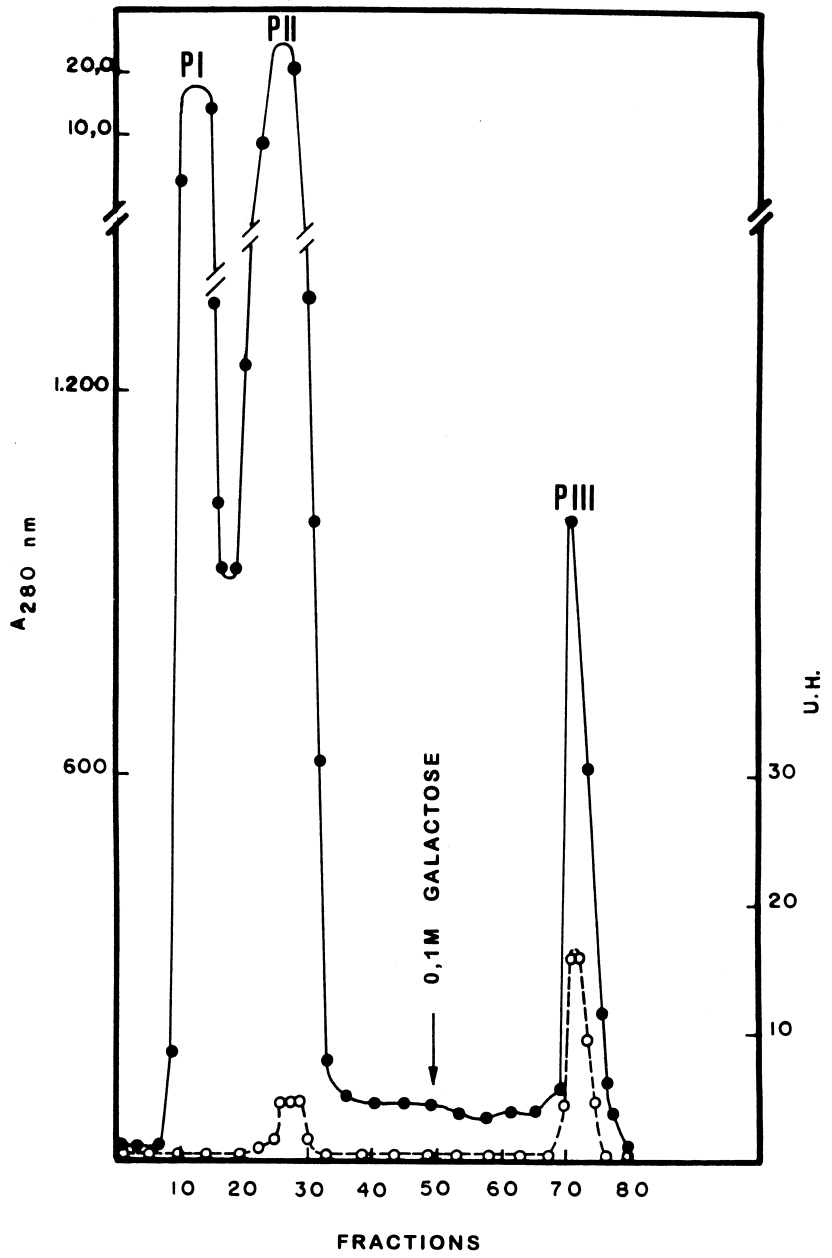

Fig. 1. Affinity chromatography on Sepharose-4B of the crude extract of *A. pulchellus*. The column was equilibrated and first eluted with 0.1 M Tris–HCl (pH 7.4) containing 0.15 M NaCl followed by the same solution containing 0.1 M galactose. Sample: 200 mg·8 ml^{-1}, flow rate: 30 $ml \cdot h^{-1}$, fractions: 2.6 ml. (– ● –) A_{280} (– ○ –) HU.

peaks was interpreted as a small contamination of lectins that usually remain in the unretained fractions during affinity chromatography techniques. The animals tested with the adsorbed fraction (PIII) often died before 24 h after intraperitoneal administration.

The PIII fraction was submitted to electrophoresis to determine the homogeneity and the molecular mass of the protein constituents (Fig. 2). Two major protein bands of Mr 63 000 and 61 500 Da were obtained in SDS-PAGE and two other of Mr 31 500 and 29 000 Da in SDS with 2-mercaptoethanol. These results fully agree with the physico-chemical properties of other toxic plant proteins with *N*-glycosidase activity (type II RIPs) which are composed of two different chains linked by a disulphide bond. (Franz, 1993; Koop *et al*., 1993). The presence of closely related toxic proteins in the PIII fraction, as observed in *A. precatorius* toxic preparations (Hegde *et al*., 1991), was investigated by gel filtration on a Superose 12HR column monitored by a FPLC system. Different proteins (or isoforms) were obtained. However, all peaks agglutinated erythrocytes and killed mice. Remarkably isoelectric focusing showed a complex pattern of protein bands with pI values varying from 6.9 to 7.7 (results not shown). Similar results have been reported for *Abrus precatorius* toxins (Hung *et al*., 1993).

Similar to other plant toxic lectins, *A. pulchellus* lectins were strongly inhibited by galactose, galactose-containing sugar and derivatives: galactosamine, α-D-melibiose, lactose and raffinose. The common galactose-specificity seems to be a very preserved characteristic from type II RIPs and the functionality of their carbohydrate-binding sites have been shown to be essential for the toxin internalisation and address into the eukaryotic cell (Lord *et al*., 1994). The antibodies anti-*A. pulchellus* toxins, fully

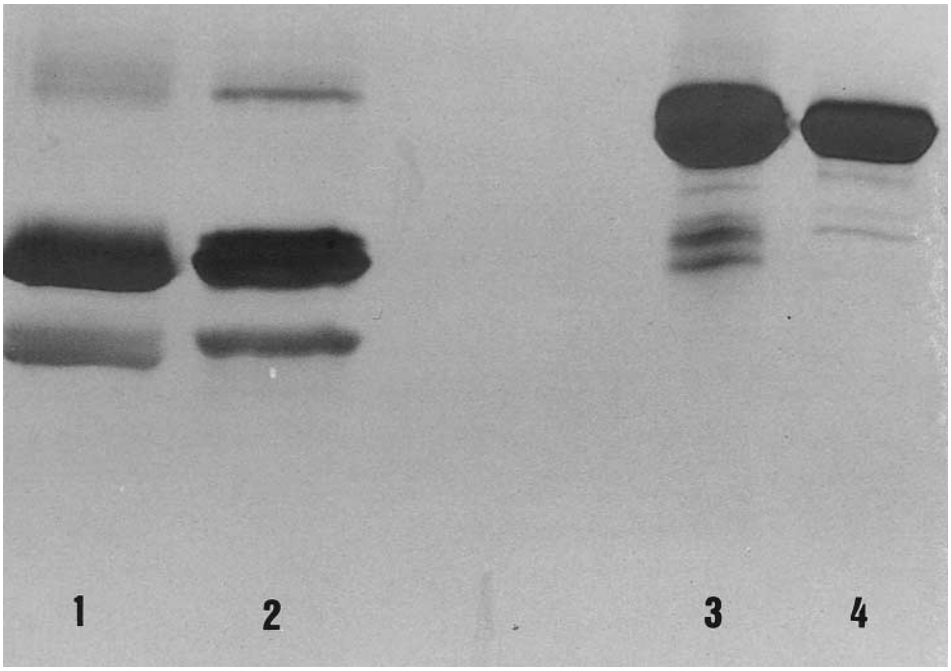

Fig. 2. Electrophoresis on 12.5% polyacrylamide gel in presence of SDS. Samples: (1) lectins from *A. pulchellus*, (2) lectins from *A. precatorius*, both isolated by affinity chromatography and reduced by 2-mercaptoethanol, (3) lectins from *A. pulchellus* and (4) lectins from *A. precatorius* not reduced.

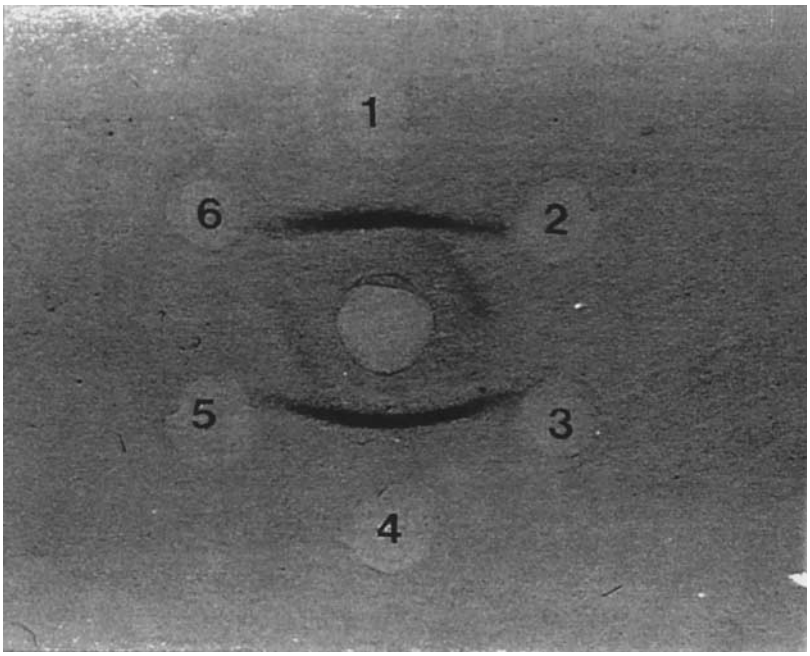

Fig. 3. Immunodifusion of antibodies anti-PIII against PIII (1 and 4), *A. precatorius* lectins (2 and 5) and *R. communis* lectins (3 and 6).

recognised *A. pulchellus* toxins, however only partial recognition was observed for *A. precatorius* toxin fraction and no ricin was recognised (Fig. 3). Although type II RIPs share many chemical, structural and functional features, it has been shown that they are not related by immunochemical properties (Olsnes *et al.*, 1974a,b; Pappenheimer *et al.*, 1974).

The toxic effect induced by the fraction PIII were investigated in order to establish its potency and the behaviour of animals after the administration of lethal and sublethal doses. Sublethal doses also lead to animal death some days later until the end of experiments. The PIII was able to kill 50% of the tested mice with doses as low as 31 $\mu g \cdot kg^{-1}$. Although this value is higher than that found for other similar toxins, the toxic effects observed agree with that induced by type II RIPs (Franz, 1993). Not surprisingly, the previous treatment of PIII with galactose (500 mM) was not capable of blocking the toxic effects as the toxins are also active in the presence of a sugar inhibitor. Similar results were achieved in the assay conducted with *Artemia salina*. Although the PIII exhibited a high toxicity ($LD_{50} = 3.5\ \mu g \cdot ml^{-1}$), samples previously exposed to lactose were equally toxic to the microcrustacean. The toxicity exhibited by the toxin–lactose complex cannot be explained in terms of metabolic removal of the sugar because it was present in large amounts. Hence, it is suggested that the toxic lectins isolated from *A. pulchellus* seed most probably recognise complex glycan in the surface of *A. saline* with a higher avidity than lactose, displaying toxic effects.

The chemical and biological properties determined in the PIII fraction suggest that this fraction contains closely related lectins which produce toxic effects similar to the ribosome inactivating proteins. This fraction is currently under investigation in order to isolate the distinct toxic lectins and establish their structural and biological character-

istics. Preliminary results indicate the presence of more than one toxin with haemagglutinating properties.

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