Purification and Characterization of Two Major Toxic Proteins from Seeds of Abrus precatorius*

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Chin Hsuan Wei,† Fred C. Hartman, Peter Pfuderer, and Wen-Kuang Yang

From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

SUMMARY

A purification procedure is described for two major toxic proteins from seeds of Abrus precatorius by successive chromatography on diethylaminoethyl-Sephadex A-50, carboxymethyl-cellulose, and DEAE-cellulose. The two toxins obtained both appeared homogeneous as judged by electrophoresis and analytical ultracentrifugation.

Abrin A, the more positively charged protein obtained from the DEAE-cellulose chromatography, can be crystallized as needles from ammonium sulfate solution by the free interface diffusion technique; under various similar conditions, the other fraction, abrin C, failed to yield crystals. X-ray precession photographs have indicated that abrin A crystallizes in an orthorhombic unit cell of symmetry P212121 and dimensions a = 74.9, b = 269.8, and c = 70.3 Å.

The amino acid composition of abrins A and C are different. Molecular weights determined by sedimentation equilibrium are 60,100 for A as compared to 63,800 for C. After A has been treated with 2-mercaptoethanol at 100° for 2 min, polycrystalline gel electrophoresis in the presence of sodium dodecyl sulfate gives rise to one strong (corresponding to apparent subunit molecular weight of 32,000) and two much weaker bands. This is in sharp contrast with the pattern for C, two bands of approximately equal intensity, corresponding to apparent subunit molecular weights of 33,000 and 28,000. The two proteins also behave differently in affinity chromatography on a Sepharcose 4B column. Abrin C at 1 µg per ml concentration level agglutinates sheep erythrocytes, whereas abrin A shows no such activity even at 40 µg per ml concentration level. Abrin C exhibits a more toxic effect on mice than abrin A. These differences in physicochemical and biological properties for the two abrins are presented in detail.

Our interest in obtaining crystals of abrin, present in the seeds of Abrus precatorius, has been stimulated by recent work concerning its anti-tumor activity (as well as that of ricin from seeds of Ricinus communis) against various sarcomas in rats and mice (1–3). Although purification procedures for abrin have been described by several authors (4–8), crystallization to usable size for x-ray studies has only recently been reported (9). Of particular interest is that Olsnes and Pihl (7) reported the isolation of abrin with about 6 times the toxicity of the most active preparation reported by Lin et al. (3–5). Apparently no correlation for the two abrin preparations previously reported by Lin et al. (3–5) and by Olsnes and Pihl (6, 7) may actually be different from each other.

In this paper, the two purified toxins have been characterized in terms of their physicochemical and biological properties, including ease of crystallization, amino acid composition, molecular weight, electrophoretic mobility, affinity to Sepharcose 4B column, hemagglutinating activity, and toxicity.

MATERIALS AND METHODS

Seeds of A. precatorius were obtained from private sources in Taiwan, Republic of China. DEAE-Sephadex A-50 (particle size 40 to 120 µm), Sepharose 4B, and Sephadex G-100 (particle size 40 to 120 µm) were purchased from Pharmacia Fine Chemicals; CM-cellulose (CM-52) and DEAE-cellulose (DE-52) were products of Whatman Co. Protein standards for sodium dodecyl sulfate electrophoresis and for protein determination were obtained from the following sources: ovalbumin, Schwarz-Mann; chymotrypsinogen, Worthington Biochemicals, Inc.; cytochrome c (horse heart), Sigma Chemical Co.; myoglobin (sperm whale), Mann Research Laboratories; and bovine serum albumin (crystallized three times), Nutritional Biochemical Co. Sheep blood for hemagglutination tests was obtained from BBL Division of Becton-Dickinson and Co. The BALB/c mice employed for toxicity tests were purchased from Cumberland View Farms, Clinton, Tenn. Ammonium sulfate (ultrapure biological grade) was purchased from Schwarz BioResearch, Inc. Other chemicals used were of analytical grade.

Protein Concentration—Spectrophotometric measurements at 280 nm were used to estimate protein concentration. The ε_max at 280 nm of purified abrins was determined as described below. Determinations by the method of Lowry et al. (10), with crystalline bovine serum albumin as the standard, were also carried out. In the early stages of our study, a value for ε_max of 12.4 given by Lin et al. (4) was utilized.

Extinction Coefficients—Samples of abrins A and C at approximately 10 and 7 mg per ml, respectively, were dialyzed exhaustively (three 1-liter changes during 72 hours) against 0.01 M ammonium acetate, pH 6.7. Abrin A was further dialyzed for 8 hours against 0.0006 N acetic acid (pH 4.4) to dissolve the precipitated protein. After centrifugation, aliquots of the supernatant were diluted 20-fold into various buffers: 0.1 M potassium phosphate,
spectively. Undiluted, dialyzed solutions of A (2 ml) and B (2.5 ml) were each lyophilized in preweighed vials, redissolved in 5 ml of water, and again lyophilized. The vials were then heated to constant weights (for 48 hours in a vacuum oven at 110°) and the el$_{280}$ at 280 nm of the samples was calculated as 14.90 and 17.74 for abrins A and B, respectively.

The protein concentrations of both abrins were also determined according to the method of Lowry et al. (10), with the use of bovine serum albumin as a standard. The albumin solutions were in turn calibrated spectrophotometrically with a value of 0.8 for el$_{280}$ at 280 nm (11). The el$_{280}$ values at 280 nm thus obtained were 11.7 for abrin A and 12.95 for abrin B. These values were used in our hemagglutination and toxicity tests.

The ratios of absorbances at 280 and 290 nm at various experimental conditions range from 1.95 to 2.00 for abrin A, and from 1.88 to 1.90 for abrin B.

Amino Acid Analyses—Protein was hydrolyzed at 110° for 24 or 72 hours in sealed, evacuated (<50 mm of Hg) tubes with 6 N HCl containing 0.01 M 2-mercaptoethanol. The hydrolysates were concentrated to dryness on a rotary evaporator and analyzed on a Beckman model 120C amino acid analyzer according to Spackman et al. (12). Insignificant differences in the amounts of most amino acids were found in comparisons of 24- and 72-hour hydrolysates. Somewhat larger quantities of valine, isoleucine, and leucine were present in the 72-hour hydrolysates, and these are not the expected. The values reported for serine and threonine are corrected for 10% and 5% destruction, respectively, during a 24-hour hydrolysis period. Total cystine and cysteine are reported as cystic acid formed during hydrolysis in the presence of dimethylaminothiourea as described by Spencer and Wold (13). Tryptophan was determined by the method of Matsubara and Sasaki (14), in which destruction of tryptophan during hydrolysis is prevented by thioglycolic acid.

Analytical Ultracentrifugation—Sedimentation equilibrium and sedimentation velocity experiments were run on a Spinco model E ultracentrifuge equipped with RITC temperature control, schlieren and interference optical systems, and adjustable optical components. All sedimentation velocity experiments were done in 12-mm 2°Kel-F centerpieces at 59,780 rpm. The equilibrium measurements were made in 12-mm double sector filled Epon centerpieces, using approximately 4-mm column heights. The meniscus depletion technique of Yphantis (15) was used. Partial specific volumes were calculated from the amino acid composition by the method of Cohn and Edsall (16) as suggested by MeMeekin and Marshall (17).

Electrophoresis—Electrophoresis experiments were carried out with analytical apparatus from Canal Industrial Corp. Polyacrylamide disc gel electrophoresis was conducted at 4° (pH 9.5) on standard 7% gels which were stained with Amido schwarz and destained electrophoretically. Polyacrylamide electrophoresis (10% gels) in sodium dodecyl sulfate was performed at room temperature (24°) by the method of Weber and Osborn (18) and Weber et al. (19). The ammonium persulfate concentration in the gel solutions was, however, decreased from the suggested value of 0.75 to 0.25 mg per ml. Gels were stained with Coomassie brilliant blue R 250 and destained electrophoretically.

Hemagglutination Test—Plasma was removed from the sheep blood in Alsever's solution, and the blood was then washed four times in sterile saline (0.9% NaCl) by centrifugation. Tests were performed in siliconized test tubes (0.5 x 7 cm) with 0.4 ml of a mixture containing 2% red blood cells, 0.14 M NaCl, 0.01 M phosphate buffer at pH 7.0, and various amounts of abrin preparations. The mixture was incubated at 37° for 30 min and was left to stand overnight at 4°. The degree of hemagglutinating activity was graded according to the pattern formed by the agglutinated cells on the bottom of the tube (20, 21). Tests were also made in 1 mg per ml of bovine serum albumin. To test the inhibition of hemagglutination, 0.075 M n-galactose was included in the mixture.

Toxicity Test—Female BALB/c mice, 4 to 5 months old and weighing 22 to 25 g, were injected intraperitoneally with 0.5 ml of nonpyrogenic sterile normal saline containing various quantities of abrin preparations. To prevent leakage of the injected solution, the 22-gauge needles were first passed subcutaneously before insertion into the peritoneal cavities of the mice. The mice were given free access to food and water, and death incidence was recorded at 12-hour intervals up to 7 days. The toxicity of a given abrin preparation was first tested by use of three groups of eight mice receiving doses of 0.1 to 1.5 µg per mouse. The LD$_{50}$ value (22) was then determined in four groups of eight mice that received a narrower dosage range of the abrin preparation. Since we observed two types of mortality, an acute type occurring within the first 2 days and a chronic type occurring over longer periods, two LD$_{50}$ values, corresponding to time periods of 48 hours and 7 days, respectively, were determined for each abrin preparation.

RESULTS

Purification of Abrins

All manipulations during purification were carried out at room temperature (24°) unless otherwise stated, and all aqueous solutions were prepared with glass-distilled water. Centrifugations were performed at 5° in a Sorvall RC-2-R centrifuge. In one case (see below) the centrifugations were performed at 10° in a Beckman L3-50 ultracentrifuge with a type 50.1 rotor.

Step 1: Extraction, Heat Treatment, and Ammonium Sulfate Fractionation—Dry seeds of A. precatorius (200 g) were crushed with a coffee grinder (Stur-de Health Products) and soaked in 1.5 liters of 5% acetic acid overnight at 4°. They were then homogenized by means of a Waring Blender. The homogenate was centrifuged in 250-ml bottles in a Sorvall centrifuge at 13,200 x g for 30 min, and the solid residue was discarded. Solid ammonium sulfate was added to the supernatant, with stirring, to 45% saturation (23). A clear liquid obtained by centrifugation at 10,400 x g for 10 hours was brought to 0° saturation with solid ammonium sulfate, and the precipitate was collected by centrifugation at 13,200 x g for 2½ hours. The pellet was dissolved in approximately 160 ml of water (with the aid of 2 ml of 0.1 N hydrochloric acid) to a pinkish, muddly, colloidal solution which was brought to 60° for 30 min with occasional swirling in a constant temperature bath. Some decoloration was noticed at this stage. After dialysis against running water for 24 hours, precipitated material was removed by centrifugation in a Beckman L3-50 ultracentrifuge at 192,000 x g for 3 hours. The yellowish transparent supernatant (360 ml) was concentrated to 110 ml by means of ultrafiltration using a PM-10 Diaflo membrane (Amicon Corp.). The light brown liquid was diluted with an equal volume of 0.01 M sodium acetate buffer (pH 5.8) and, after mixing, the pH was readjusted to 5.8 with a small amount of 1 N sodium hydroxide. The resulting solution is designated the crude extract.

Step 2: DEAE-Sephadex A-50 Chromatography—The crude extract was passed over a column of DEAE-Sephadex A-50 which was eluted with a buffer containing 0.005 M sodium acetate (pH 5.8). The proteins usually appeared in a rather irregular broad peak (Fig. 1a). Examination of various fractions of this broad peak showed no apparent differences in toxicity for mice. An average LD$_{50}$ value at 48 hours was estimated to be 1.5 µg of protein per mouse. Fractions having A$_{280}$ values greater than 0.25 were pooled as shown in Fig. 1a and concentrated by means of the ultrafiltration or pervaporation technique (or both). After dialyzing against running water for 12 hours, the concentrated solution was diluted with an equal volume of 0.02 M sodium phosphate buffer, pH 5.8, to yield approximately 100 ml of solution with an A$_{280}$:A$_{250}$ ratio of 1.40.

Step 3: CM-cellulose Chromatography—Because of the size of column employed, the solution obtained in Step 2 was divided into two equal amounts for separate runs. Each sample, con-
FIG. 1. a, DEAE-Sephadex A-50 chromatography at room temperature (24°C). The column (5 X 76 cm) was pre-equilibrated with a buffer containing 0.005 M sodium acetate (pH 5.8), which was also used to elute the proteins with an initial flow rate of 2.6 ml per min. The eluted protein appeared as a broad irregular peak at the beginning, followed by a peak containing greenish, low molecular weight material which was discarded. b, CM-cellulose chromatography at room temperature (24°C). The column (2.5 X 40 cm), pre-equilibrated with 0.01 M sodium phosphate buffer (pH 5.8), was first eluted with the same buffer, and then with a linear gradient of NaCl (0.05 to 0.5 M) in the phosphate buffer. c, DEAE-cellulose chromatography at room temperature (24°C). The experiment was carried out on a column (2.5 X 39 cm) with a gradient elution of potassium phosphate buffer (0.01 to 0.28 M), pH 7.7. Two major fractions were designated abrin A and C, and one very minor fraction was designated Peak B.

taining approximately 460 mg of protein, was introduced on a CM-52 column which was first washed with 0.01 M sodium phosphate buffer (pH 5.8) to remove all elutable materials which were nontoxic (no death at the level of 10 µg per mouse). The protein was then eluted with a linear gradient of sodium chloride (0.05 to 0.5 M, 1 liter each) in the phosphate buffer (Fig. 1b). The LD₅₀ values of the pooled fractions were determined to be 0.6 µg per mouse at 48 hours and 0.2 µg per mouse at 7 days for 6-month-old mice. The eluted toxins were precipitated with solid ammonium sulfate, dissolved in 40 ml of water, dialyzed against water, concentrated, and finally dialyzed against 0.01 M potassium phosphate buffer (pH 7.7) for the following step. Approximately 250 mg of proteins were obtained in 45 ml from one batch.

Step 4: DEAE-cellulose Chromatography—The toxins were further purified by chromatography on a DE-52 column. As shown in Fig. 1c, proteins were separated with a gradient elution of potassium phosphate buffer (0.01 to 0.28 M, 1 liter each), pH 7.7. The major fractions, designated Abrin A and C (105 and 104 mg, respectively), and one very minor fraction, designated Peak B, were precipitated with ammonium sulfate and dialyzed against water. The analyses of physicochemical and biological properties, described below, were carried out principally for the major fractions A and C.

Characterization of Abrins A and C

Homogeneity—Abrin A sediments as a single boundary whereas abrin C seems to contain a small amount of low molecular weight material in the analytical ultracentrifuge, schlieren patterns from which are shown in Fig. 2, a and b. Examination of the concentration-dependence of the sedimentation constants yields average values of 4.62 ± 0.05 S for abrin A and 4.65 ± 0.05 S for abrin C, both at infinite dilution. As shown in Fig. 2, d and e, abrin A and C each migrate as a single band during disc gel electrophoresis. The minor component B (Fig. 1c), which persistently appeared in the DE-52 runs, was also found in disc gel electrophoresis to travel as a single band with a velocity between those of abrins A and C.

Crystallization—There is a remarkable difference in crystallization properties for the two abrins. On standing for a few weeks, abrin A usually deposits out as fine crystals from either 0.2% aqueous solution or from the eluates of the DE-52 chromatography, which contain about 0.2 to 0.3% protein in the region of 0.015 M potassium phosphate buffer (pH 7.7) solution, while abrin C in similar solutions yields an amorphous precipitate, if any. When the aqueous solution of abrin A is dialyzed against 0.005 to 0.01 M sodium phosphate buffer at pH 6.8 (with or without the presence of cupric sulfate), rod-shaped crystals up to 0.05 mm long usually appear within 2 to 3 weeks; under similar conditions abrin C failed to yield crystals. Crystallographi-
Table I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Abrin A</th>
<th>Abrin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>2.2</td>
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<td>Histidine</td>
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<td>1.0</td>
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<td>Arginine</td>
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<td>Cysteic acid</td>
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<td>Aspartic acid</td>
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<td>4.3</td>
</tr>
<tr>
<td>Serine</td>
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</tr>
<tr>
<td>Glutamic acid</td>
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<td>8.4</td>
</tr>
<tr>
<td>Proline</td>
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<td>3.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.9</td>
<td>6.2</td>
</tr>
<tr>
<td>Alanine</td>
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<td>4.9</td>
</tr>
<tr>
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<td>4.9</td>
</tr>
<tr>
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<td>1.9</td>
</tr>
<tr>
<td>Isoleucine</td>
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</tr>
<tr>
<td>Leucine</td>
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<td>6.5</td>
</tr>
<tr>
<td>Tyrosine</td>
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<td>4.1</td>
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<tr>
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</tr>
<tr>
<td>Tryptophan</td>
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<td>1.1</td>
</tr>
</tbody>
</table>

* These values were calculated from the data of Lin et al. (5) after correcting for the fact that they reported the number of residues per 27,350 daltons instead of the number of residues per 32,500 daltons as stated.

Amino Acid Composition—The amino acid compositions of abrins A and C are given in Table I, together with that of the crystalline abrin preparation reported by Lin et al. (5) for the purpose of comparison. By setting the histidyl content to one residue per mole, minimal molecular weights of 6850 and 9600 are calculated for abrins A and C, respectively.

Molecular Weight—The molecular weight of abrin A, as estimated by sedimentation equilibrium, is 60,100 ± 1,000 and that of abrin C is 63,800 ± 2,000. Data from one experiment on each protein are shown in Fig. 3. Although abrin C appeared to contain small amounts of low molecular weight material in schlieren photographs of the velocity sedimentation experiments (Fig. 2b), no sign of this sort of material is seen in the equilibrium experiment in Fig. 3b. The equilibrium experiments were carried out at five different speeds ranging from 16,200 to 24,630 rpm resulted in essentially the same molecular weight for abrin C. Gel electrophoresis experiments also failed to give any evidence for low molecular weight material (Figs. 2e and 4d). It is possible that abrin C was dissociating under the conditions of

Fig. 3. Molecular weight runs of abrins A and C as obtained from equilibrium sedimentation data. The proteins were in 0.1 M NaCl-0.005 M sodium acetate buffer, pH 4.7. a, abrin A at a protein concentration of 0.12 mg per ml was centrifuged at 24,630 rpm for 33 hours at 12.5°. b, abrin C at a concentration of 0.40 mg per ml was centrifuged at 24,630 rpm for 30 hours at 15°. In the figures, r represents radius and t represents fringe displacement concentration measured in microns. Molecular weights were obtained from the least squares slope of the corresponding plots (15).

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of abrins A and C, each without (a and d) and with (b and e) the 2-mercaptoethanol treatment at 100° in 2 min. In c, abrin A was treated with performic acid before electrophoresis. Direction of migration is toward bottoms of tubes (anodes).
the velocity experiments, or that a low molecular weight impurity was present in our preparation, in which case the molecular weight estimate of 63,800 would be lower than the true value. With $v$ values of 0.734 and 0.731 ml per g, and $s_{20w}$ values of 4.62 and 4.65 S, respectively, for abrins A and C, diffusion constants are calculated to be $7.0 \times 10^{-14}$ and $6.6 \times 10^{-14}$ cm$^2$ s$^{-1}$, respectively, at 20$^\circ$.

Electrophoresis Pattern on Sodium Dodecyl Sulfate Polyacrylamide Gel—When abrins were treated with 2-mercaptoethanol at 100$^\circ$ for 2 min (19) before electrophoresis, abrin A gave rise to one strong and two weak bands in contrast with the pattern obtained for abrin C, which showed two bands of approximately equal intensity. When abrin A was oxidized with performic acid prior to electrophoresis according to method 3 (19), only a single, somewhat diffuse band was observed. The patterns obtained with and without 2-mercaptoethanol treatment are given in Fig. 4. No difference in pattern was detected between samples before and after the Sepharose 4B chromatography (see below) although the results of affinity chromatography indicated a trace of impurity present in each case.

With proteins of known molecular weights as markers (Fig. 5), the molecular weight of the major component for abrin A was estimated to be 32,000 and those of the minor components were 29,500 and 28,000 (see below). The molecular weights of abrin C subunits were estimated to be 33,000 and 28,000 (all & 1,500).

Affinity Chromatography—The different binding affinity of the two abrins to Sepharose 4B was examined by chromatography at 4$^\circ$. Abrin A, eluted with 0.01 M Tris-HCl buffer (pH 7.8) containing 0.03 M sodium chloride, gave rise to a broad peak which was preceded by approximately 3% of some impurity (Fig. 6a). Since further elution of the column with 0.1 M d-galactose-containing buffer did not result in any additional peak, this signified that abrin A was loosely bound to the column. In the case of abrin C, a small amount (about 1.5%) of material was eluted by the first 100 ml of buffer. The toxin was eluted as a single sharp peak with the same buffer containing 0.1 M d-galactose (Fig. 6b). This indicates that abrin C was strongly bound to the column under the experimental conditions.

With the use of a spectrophotometer (Gilford Instrument model 240 measured at 660 nm), the integrated intensity ratios for the three bands of abrin A were estimated to be 8:2:1, while the ratio for the two bands of abrin C was 5:4. In each case, values are for bands of increasing order of migration.

Fig. 5. Subunit molecular weight determinations of abrins A (□) and C (△) by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecular weights of the subunits of the marker proteins are: ovalbumin, (□), 45,000 (average value designated by the maker); chymotrypsinogen, (●), 26,700 (19); myoglobin (sperm whale), (○), 17,190 (20); and cytochrome c (horse heart), (△), 13,400 (see Ref. 11, p. C-13). (Abrins A and C without 2-mercaptoethanol are also shown as □ and △, respectively.)

Fig. 6. Affinity chromatography of abrins A and C on Sepharose 4B columns (1.5 X 20 cm). In each case, approximately 20 mg of protein in 10 ml of 0.1 M Tris-HCl buffer (pH 7.8), containing 0.03 M sodium chloride, were first adsorbed on the column which was pre-equilibrated with the buffer. After 2 hours, the column was eluted with the same buffer. Abrin A was eluted as a broad peak (a), and further elution of the column with 0.1 M d-galactose-containing buffer did not result in any additional peak. Abrin C was first eluted with 100 ml of the plain buffer, followed by the elution with 0.1 M d-galactose-containing buffer to give rise to a single sharp peak (b). The experiments were performed at 4$^\circ$ at a flow rate of 23 ml per hour.

### Table II

<table>
<thead>
<tr>
<th>Protein concentration</th>
<th>Abrin A</th>
<th>Abrin C</th>
</tr>
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<tbody>
<tr>
<td>µg/ml</td>
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<td>N.D.</td>
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<tr>
<td>40.00</td>
<td>(--)</td>
<td></td>
</tr>
<tr>
<td>20.00</td>
<td>(--)</td>
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</tr>
<tr>
<td>10.00</td>
<td>(--)</td>
<td>N.D.</td>
</tr>
<tr>
<td>5.00</td>
<td>(--)</td>
<td>±(±)</td>
</tr>
<tr>
<td>2.50</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>1.25</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>0.63</td>
<td>(--)</td>
<td>±(--)</td>
</tr>
<tr>
<td>0.31</td>
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<td>(--)</td>
</tr>
<tr>
<td>0.16</td>
<td>(--)</td>
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</tr>
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</table>

* Purified preparations from Sepharose 4B chromatography (Fig. 6).

b Scoring of the reaction was as follows: +, diffuse film of agglutinated cells covering the bottom of the tube; ±, diffuse film of agglutinated cells covering a smaller area of the bottom of tube; −, discrete red button of cells in center of bottom of tube (20, 21). Scores in parentheses were obtained with the reaction mixtures each containing 0.075 M of d-galactose.

* Not determined.

a binding which was abolished by d-galactose in a manner similar to that found by Olsnes and Pihl (6) for their abrin preparation.

Hemagglutination—The two abrins showed a considerable difference in their ability to agglutinate sheep red blood cells (Table II). At concentrations below 40 µg per ml, abrin A did...
not give agglutination, whereas abrin C was able to cause agglutination at 1 µg per ml concentration level. Addition of 1 mg per ml of bovine serum albumin in the reaction mixture did not significantly increase the hemagglutination titer of the two abrins.

The agglutination by abrin C, with concentration less than 10 µg per ml, could be inhibited when 0.075 M of β-galactose was included in each of the reaction mixtures.

**Toxicity**—Both abrins A and C prepared from the DEAE-cellulose chromatography (Fig. 1c) were highly toxic to the BALB/c mice by intraperitoneal injection (Table III). The animal showed the symptoms of lusterless hair and general weakness 12 hours after injection, and death occurred within approximately 48 hours. The LD₅₀ per mouse for this acute type of mortality of the 3- to 5-month-old female BALB/c mice was 0.6 µg for abrin A and 0.2 µg for abrin C. The mice which received dosages close to these values and survived beyond 48 hours, however, showed symptoms such as anorexia, diarrhea, and cachexia, and some died between 4 and 7 days after injection. Based on the cumulative incidence of death at 7 days, the LD₅₀ values were considerably lower, 0.25 µg for abrin A and 0.07 µg for abrin C. Thus, abrin C appeared to be 3- to 4-fold more toxic than abrin A. Autopsy of those dead at 48 hours (acute type) demonstrated profuse ascites, edematous and hemorrhagic appearance of the viscera, and histologically coagulative cell death in the surface layer of organs which had had direct contact with the injected abrin solution. Autopsy of those dead at 7 days (chronic type) revealed an extensive adhesion of the intestines, general venous congestion, and severely depleted cellularity with many pyknotic cells in the spleen. Abrins A and C caused generally similar pathological manifestations, except that local reactions seemed more severe with abrin A, and that cell depletion in the spleen seemed more marked with abrin C. Although abrin C was found to possess a strong affinity for galactosyl groups, as demonstrated by agarose-affinity chromatography (Fig. 6b), injection of this toxin in 1% β-galactose solution offered no protection of the mice against either the acute or the chronic types of toxicity. Further purification of the DE-52-isolated abrins A and C by the agarose-affinity chromatography did not decrease the LD₅₀ nor alter the toxic properties of these proteins. Determination of the LD₅₀ of the abrins at 48 hours was strongly affected by the age of the animal used. Young mice (6 weeks old) showed a relative susceptibility to acute (48 hour) mortality but no significant difference in cumulative mortality at 7 days.

**Discussion**

Although repeated attempts were made to isolate and purify abrin from seed kernels of *A. precatorius* according to the method described by Lin et al. (4, 5), our results seemed to indicate that a single chromatographic separation step with DEAE-Sephadex A-50 does not remove lectins or all brownish impurities present in the crude extract. In one case, after keeping the sample at 4° for several days, crystalline abrin appeared in a brown tinted, viscous eluate which seemed to be still contaminated with some impurities. In a recent study, Olsnes and Phl (7) reported the isolation of abrin by an alternative procedure and characterized it in terms of many properties, including toxicity, subunit structure, and affinity to Sepharose 4B column. Our finding that *A. precatorius* contains at least two major proteins raises the possibility that the previously reported preparations are not identical. We have therefore attempted to characterize the two toxic proteins that we have isolated in a manner that will permit relating them to those described in the literature.

| Table III |
|---|---|---|---|---|
| Experiment* | Age of mice | Abrin A (µg/mouse) | Abrin C (µg/mouse) |
|   | 48 hrs | 7 days | 48 hrs | 7 days |
| I  | 3-5    | 0.75  | 0.38  | 0.35  | 0.10  |
| II | 1.4 (6 weeks) | 0.45  | 0.25  | 0.12  | 0.07  |
| III | 3-5    | 0.60  | 0.25  | 0.20  | 0.07  |
| IV | 4      | 0.65  | 0.20  | 0.25  | 0.08  |

* In Experiments I, II, and III preparations from three different runs of DEAE-cellulose chromatography were used.

* Abrin injected in a solution of 1% β-galactose.

In our purification procedure, a DEAE-Sephadex A-50 column was used to filter off most of the brown colored impurities. We observed that the contaminating nontoxic viscous impurities with an A₂₈₀/A₂₈₀ ratio ranging from 0.9 to 1.2 can be removed by the use of the CM-cellulose chromatography. The mixture of toxins eluted as a single, but slightly asymmetrical peak (Fig. 1b), a pseudohomogeneity which persisted when the sample was passed through a Sephadex G-100 column (2.5 x 94 cm) with a buffer (pH 6.8) containing 0.05 M sodium phosphate and 0.1 M sodium chloride. Although crystals of up to 0.05 mm can be grown from this material by dialyzing the solution against 0.005 to 0.01 M sodium phosphate buffer (pH 6.8), a disc gel electrophoresis experiment revealed two distinct bands (Fig. 2a). The fact that this mixture cannot be separated by gel filtration suggested that the two components have similar molecular weights, a conclusion which was later verified by analytical ultracentrifugation.

Separation of the two toxins was achieved by DEAE-cellulose chromatography, from which two major fractions (with varying relative amounts) were obtained (Fig. 1c). Each fraction appeared homogeneous as judged by sedimentation velocity (Fig. 2a and b) and by electrophoretic mobility (Fig. 2e, d and e, and Fig. 4a, a and d).

As calculated from equilibrium ultracentrifugation, the molecular weights of abrins isolated in the present study are 60,100 for abrin A, and 63,800 for abrin C. Based on the minimal molecular weights determined from the amino acid compositions, molecular weights of 62,000 and 67,000 can be calculated for abrins A and C, respectively. However, since the minimal molecular weights are so small, the calculated molecular weights based on the amino acid compositions are not particularly reliable. These calculated molecular weights for abrins A and C are close to each other, and neither is much different from the value of 65,000 reported by Lin et al. (3, 5) and Olsnes and Phl (6, 7) for their abrin preparations. The importance of characterizing the two abrins in our study by other physicochemical or biological properties (or both), therefore, cannot be over-estimated.

As shown in Fig. 4b, the polyaerylamide gel electrophoresis pattern in sodium dodecyl sulfate for abrin A (after treating the sample at 100° for 2 min) revealed that there are one strong and two much weaker bands, in sharp contrast with the pattern of two bands of approximately equal intensity for C (Fig. 4e). This pattern for abrin A, giving rise to apparent subunit molecular weights of 32,000, 29,500, and 28,000 (Fig. 5), does not provide unambiguous information regarding its subunit structure.

When the performic acid oxidation method was applied to the
sample prior to electrophoresis, however, only a single, somewhat diffuse band was observed at the position corresponding to molecular weight of 31,000 (Fig. 4c). Therefore a possible explanation regarding the subunit structure of abrin A is that abrin A contains two similar size subunits of 32,000 molecular weight and that the minor components of slightly smaller size observed in Fig. 4b are due to proteolytic degradation during the preparation of the samples. It is also possible that the component present in the least amount is a contamination that was not detected by electrophoresis and analytical ultracentrifugation experiments for the undissociated material. The pattern for abrin C (Fig. 4e), on the other hand, shows that the protein molecule is converted into two subunits by 2-mercaptoethanol, in agreement with the finding of Olsnes and Pihl (6) for their preparation. While a vexing discrepancy in calculated subunit molecular weights between our data (33,000 and 28,000) and those of Olsnes and Pihl (35,000 and 30,000) remains, it should be pointed out that the two peptide bands in our experiments move at the velocity ratio of 1.180 in accord with 1.165 given by the same authors.

One of the most salient features distinguishing the two abrins isolated in the present study from one another is their binding affinity to Sepharose 4B under our experimental conditions. It is clear (Fig. 6) that abrin A is loosely bound to the column and can be eluted without the D-galactose-containing buffer, whereas abrin C is tightly bound to the column, a binding that can be abolished by D-galactose. This important property of abrin C is consistent with that described by Olsnes and Pihl (7), thereby further suggesting the identity of abrin C with their abrin preparation.

It has been suggested in earlier literature (see Refs. 7 and 26, and references cited therein) that the toxic effect of abrin might be due to the presence of the hemagglutinin present in the seeds of A. procatorius. Examination of Table II reveals that abrin A contains no hemagglutinating activity (up to the protein concentration of 40 μg per ml) to sheep erythrocytes. This result may be compared with that of Lin et al. (5) who showed negative hemagglutinating activity for their abrin preparation to human group O blood cells and suggests that hemagglutinating activity is not the sole factor attributable to the toxic effect of abrin A. Abrin C, on the contrary, does agglutinate the sheep erythrocytes at the minimum concentration of 0.63 μg per ml. This activity of abrin C, however, is considerably weakened in the presence of D-galactose, a consequence presumably caused by a parallel competitive reaction between the hemagglutinating activity and its affinity to be bound to D-galactose (see above).

Abrins A and C are different by a number of criteria noted above. Several properties of abrin C strongly suggest that it may be identical with the preparation of Olsnes and Pihl (7). It is therefore highly tempting to speculate that abrin A may be identical to the crystalline preparation of Lin et al. (4, 5). Although there is much less direct evidence for comparison,5 the existing evidence does not contradict this hypothesis. The amino acid compositions (Table I) and molecular weights are similar.4 Just as abrin A is less toxic than abrin C, the preparation of Lin et al. is less toxic than that of Olsnes and Pihl (7). Abrin A, but not abrin C, can be crystallized under conditions similar to the crystallization of the preparation of Lin et al. (5). Although it seems likely that Lin’s chromatographic product contained abrin C as well as abrin A, one may speculate that only A crystallized out, giving a homogeneous material in their disc gel electrophoresis and in analytical ultracentrifugation (5).

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*Neither the sodium dodecyl sulfate polyacrylamide electrophoresis pattern nor the affinity behavior to Sepharose 4B have been published for the preparation of Lin et al.*

*The most significant differences in the amino acid composition of abrin A and the preparation of Lin et al. are in the basic amino acids. However, the internal ratios of histidine to lysine to arginine are essentially identical (2:1:4) for the two materials, so that the difference seen in comparing the number of residues per mole may reflect an error in relating data from the short column to those from the long column.*
Purification and Characterization of Two Major Toxic Proteins from Seeds of *Abrus precatorius*

Chin Hsuan Wei, Fred C. Hartman, Peter Pfuderer and Wen-Kuang Yang


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