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ISOLATION OF FOUR ISOTOXIC PROTEINS AND ONE AGGLUTININ FROM JEQUIRITI BEAN (*ABRUS PRECATORIUS*)

JUNG-YAW LIN, TE-CHANG LEE, SU-TIENG HU and TA-CHENG TUNG

Institute of Biochemistry, College of Medicine, National Taiwan University, Republic of China

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J.-Y. LIN, T.-C. LEE, S.-T. HU and T.-C. TUNG. Isolation of four isotoxic proteins and one agglutinin from jequiriti bean (*Abrus precatorius*). *Toxicon* **19**, 41–51, 1981. — Four isotoxic proteins and one agglutinin were purified from the seeds of *Abrus precatorius* by Sepharose 4B and DEAE-cellulose column chromatography. Abrin-b and abrin-c which bind weakly on the Sepharose 4B are eluted from the column in the absence of galactose while abrin-a, abrin-d and abrus agglutinin which bind strongly are eluted with 0·1M galactose. The mol. wts of abrin-a and abrin-c are 63,000 and abrin-b, abrin-d and abrus agglutinin 67,000. The lethality of the four abrins was similar; 10, 25, 16 and 31 μg/kg body wt for abrin-a, b, c and d, respectively. The amino acid compositions of four isotoxic proteins and the A and B subunit of three isotoxic proteins are similar but not identical, and they are different from those of abrus agglutinin.

INTRODUCTION

SEEDS of *Abrus precatorius* contain lectins and toxic proteins which have a strong inhibitory effect on the growth of Ehrlich ascites tumor cells (BOYD, 1970; LIN *et al.*, 1970). The isolation of lectins and toxic proteins has been achieved by several laboratories (MCPHERSON and RICH, 1973; WEI *et al.*, 1974; OLSNES *et al.*, 1974; ROY *et al.*, 1976). Recently, two abrins with different hemagglutinating activity and galactose binding activity have been reported (LIN *et al.*, 1978). The present paper describes four toxic proteins (abrins-a, b, c, d) which have been purified and their physico-chemical properties characterized.

MATERIALS AND METHODS

Materials

DEAE-cellulose was obtained from Whatman Co., Kent, England. Sepharose 4B, Sephadex G–100 (particle size 40–120 μm) and Sephadex G–150 (particle size 40–120μm) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All chemicals were of analytical grade. Seeds of *A. precatorius* were obtained from farmers who collected the bean in the southern part of Taiwan, Republic of China.

Preparation of crude extracts

Abrins were extracted by homogenizing 200 g of *A. precatorius* kernels in 1 liter of cold 5% acetic acid and the homogenate was centrifuged at 10,000 g for 20 min. The supernatants were fractionated with ammonium sulfate and the proteins which precipitated between 35 and 95% of ammonium sulfate were collected and dialyzed for 36 hr against 0·005M phosphate buffer, pH 8·0, containing 0·001M EDTA. After dialysis, the extracts were centrifuged at 10,000 g for 20 min, and the supernatants were referred in the following as crude extracts.

Hemagglutination

To 0·2 ml of 1% washed human O-type red blood cells in 0·01M phosphate buffer (pH7·4) containing 0·14M NaCl, various concentrations of abrins were added. The reaction mixtures were kept at room temperature for 2 hr. The degree of hemagglutinating activity was recorded according to the pattern formed by agglutinated cells on the bottom of the tubes (STAVITSKY, 1954).

Lethality

Male Swiss white mice, weighing 20±2 g, were injected i. p. with 0·2 ml N saline containing various amounts of abrin. After 48 hr observation, the LD_{50} value was calculated as described by REED and MUENCH (1938).

Separation of subunits and electrophoresis

To separate the A and B subunits of abrins, the isotoxin was dissolved in 8M urea–0·01M Tris–HCl, pH 8·6, and reduced by addition of 5% 2–mercaptoethanol at room temperature for 2 hr. The reaction mixtures were then applied to a Sephadex G–150 column (1·8 × 90 cm) equilibrated with 0·01M Tris–HCl (pH 8·6) containing 6M urea and 1% 2–mercaptoethanol, and eluted with the same buffer. The mol. wts of isotoxins and their subunits, A and B chains, were measured by polyacrylamide gel electrophoresis (PAGE) containing 0·1% sodium dodecyl sulfate (SDS) (KOSTRABA *et al.*, 1975).

Amino acid analysis and carbohydrate determination

Proteins were hydrolyzed at 105°C for 24 hr in sealed and evacuated tubes with 6N HCl containing 5 μl of 2–mercaptoethanol. The hydrolyzates were concentrated to dryness with an Evapo–Mix and analyzed on a Beckman model 120C amino acid analyzer according to the method of SPACKMAN *et al.* (1958). The contents of hexose in the isotoxins and abrus agglutinin were determined by the method of WINZLER (1955), and those of glucosamine were analyzed on the short column of the amino acid analyzer after hydrolysis with 6N HCl (GATT and BERMAN, 1966). Mannose and galactose (1:1, w/w) were used as standard sugars.

RESULTS

Isolation of four abrins and one agglutinin

Crude extracts (see Materials and Methods) were applied on a Sepharose 4B affinity column pre-equilibrated with 0·005M phosphate bufer, pH 8·0, containing 0·2M NaCl. The column was first washed with the buffer saline to elute the proteins which were not absorbed on the Sepharose 4B and the proteins which were toxic and weakly absorbed on the column, and then with 0·1M D(+)-galactose to elute the toxic and strongly absorbed proteins. As shown in Fig. 1, three protein peaks were obtained. The second and the third peaks were toxic but not pure as judged by PAGE while the first peak was non-lectin and non-toxic.

The materials in the second and the third peaks were further purified by chromatography with a DEAE-cellulose column. As shown in Fig. 2, two major peaks were eluted from the second peak of Fig. 1, designated as abrin-b and abrin-c; the yield was 25 and 35 mg for abrin-b and abrin-c, respectively.

Figure 3 shows that the third peak of Fig. 1 can be further separated into two peaks. The first peak which was shown to be homogeneous by PAGE was designated as abrin-d. The yield of abrin-d was 1 mg. The second peak which was demonstrated to be heterogeneous

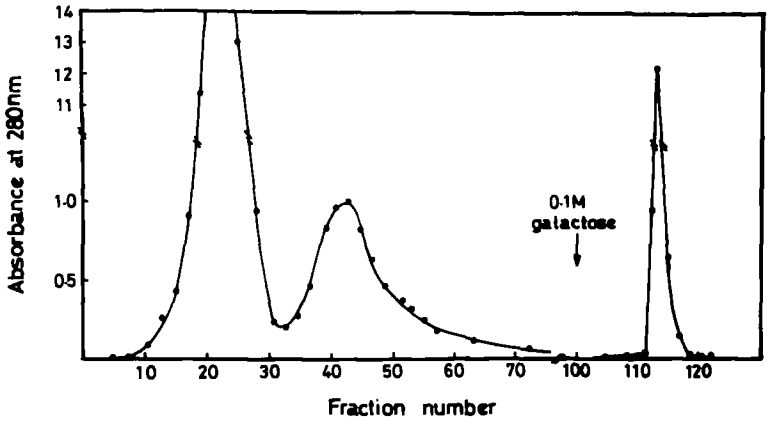

FIG. 1. AFFINITY CHROMATOGRAPHY ON A SEPHAROSE 4B COLUMN.
The crude extracts were applied to a Sepharose 4B column (3 × 45 cm) equilibrated with 0·005M phosphate buffer, pH 8·0, containing 0·2M NaCl. The column was first eluted with the above buffer and two peaks were obtained. Then it was eluted with 0·1M galactose in the above buffer, and the third peak was obtained. The flow rate was 40 ml/hr, and the volume of each fraction was 15 ml.

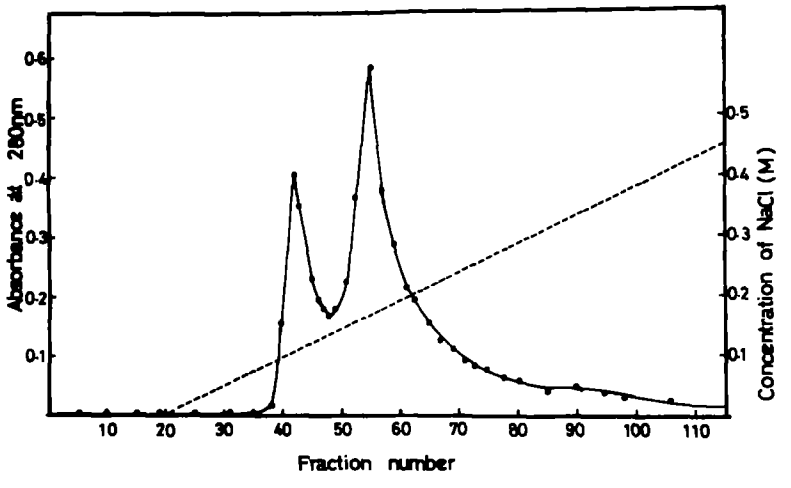

FIG. 2. DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF ABRIN-b AND ABRIN-c.
The proteins from the second peak of Fig. 1 were applied on a DEAE-cellulose column (2 × 15 cm) equilibrated with 0·01M Tris buffer, pH 8·6. Two peaks were eluted with a NaCl gradient (0 – 0·5M, 200 ml of each) in the above buffer at a flow rate of 90 ml/hr. The first peak was designated as abrin-b and the second as abrin-c. The volume of each fraction was 3 ml.

was further purified by Sephadex G–100 column chromatography. As shown in Fig. 4, two peaks were detected; the first peak was designated as abrus agglutinin while the second one was designated as abrin-a. The yield of abrin-a or abrus agglutinin was 200 mg.

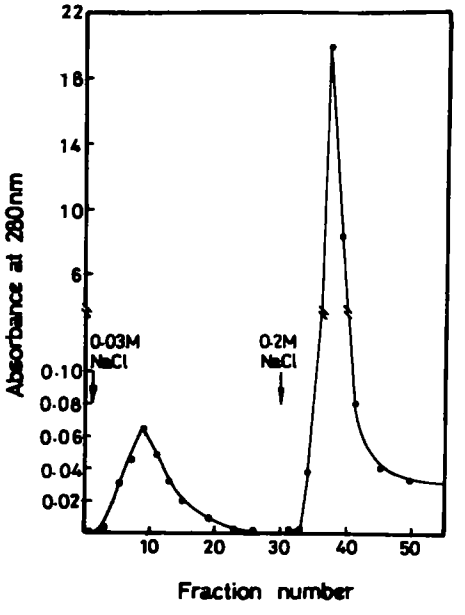

FIG. 3. DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF ABRIN-d.
The proteins from the third peak of Fig. 1 were applied on a DEAE-cellulose column (2 × 15 cm) equilibrated with 0·01M Tris buffer, pH 8·6. It was eluted with 0·03M NaCl in 0·01M Tris buffer, and one protein peak was obtained and designated as abrin-d. Then it was eluted with 0·2M NaCl in the same buffer, and one protein peak was obtained, which contained two proteins.

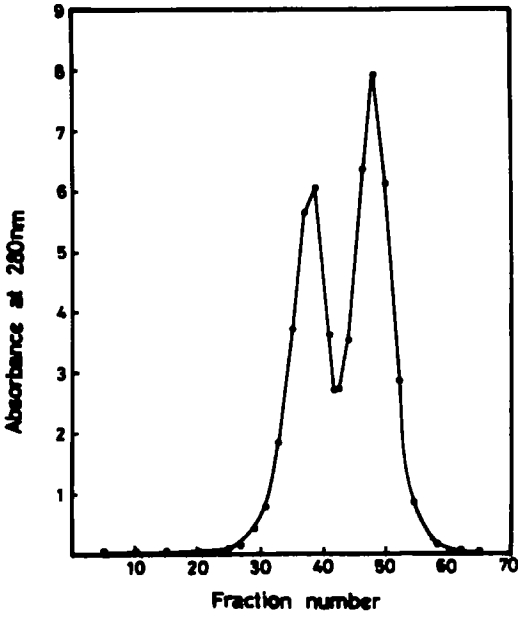

FIG. 4. SEPHADEX G–100 COLUMN CHROMATOGRAPHY OF ABRIN-a AND ABRUS AGGLUTININ.
The proteins from the second peak of Fig. 3 were applied to a Sephadex G–100 column (2·5 × 100 cm) pre-equilibrated with 0·005M phosphate buffer, pH7·2. It was eluted with the above buffer. Two peaks were obtained; the first was identified as abrus agglutinin while the second was abrin-a. The volume of each fraction was 4 ml.

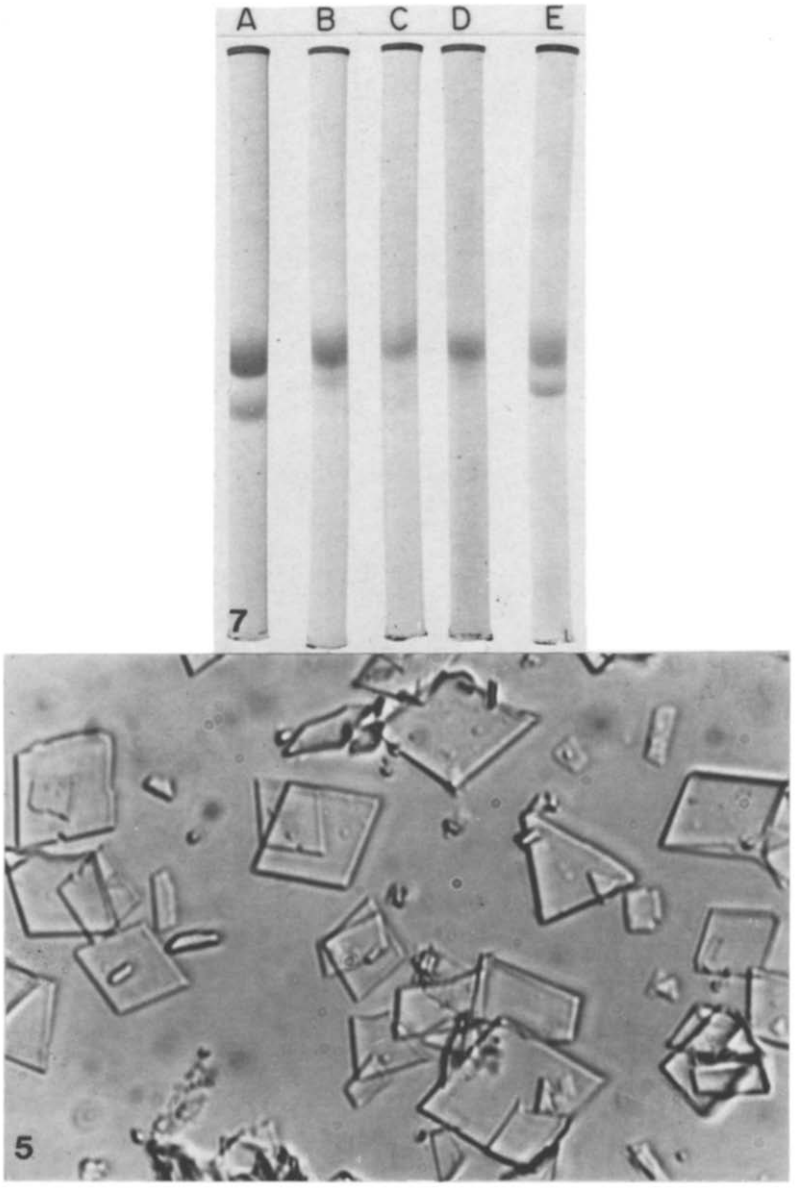

FIG. 5. CO-CRYSTALS OF ABRIN-b AND ABRIN-c (400 ×).
The method of crystallization was described under experimental procedures.

FIG. 7. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS.
Abrin-a, b, c, d or abrus agglutinin was treated with 1% SDS in the presence of 5% mercaptoethanol, and analysed by PAGE in a 12·5% gel containing 0·1% SDS. (A) abrin-a, (B) abrin-b, (C) abrin-c, (D) abrin-d and (E) abrus agglutinin.

Crystallization

When the second peak from the Sepharose 4B column (Fig. 1) chromatography containing abrin-b and abrin-c was concentrated by precipitation with ammonium sulfate and dialyzed against distilled water, the rhombic shaped crystals (Fig. 5) appeared within 1–2 days. Under the same conditions abrins-a, b, c and d failed to give crystals.

Separation of subunits

After reduction with 2–mercaptoethanol in the presence of 8M urea, the reaction products were fractionated by gel filtration with a Sephadex G–150 column (Fig. 6). Two peaks were detected; the first one was the A chain whereas the second one was the B chain judged by PAGE and amino acid composition analysis.

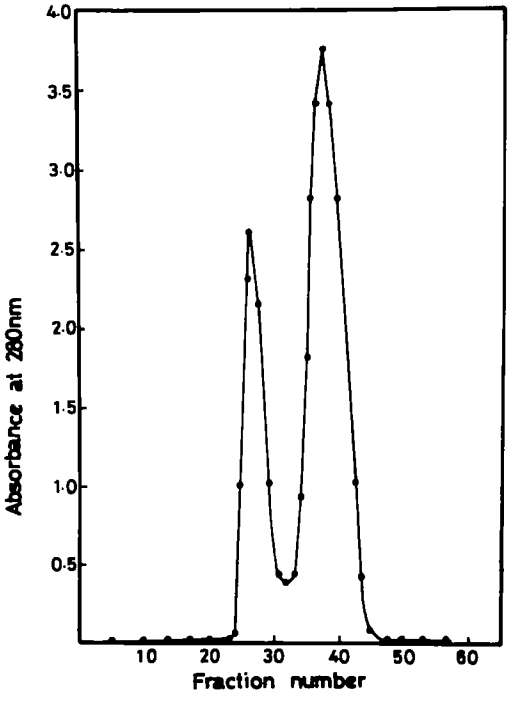

FIG. 6. SEPARATION OF SUBUNITS OF ABRINS ON A SEPHADEX G–150 COLUMN.
The protein (100 mg) was reduced by 5% 2–mercaptoethanol in 8M urea, and then applied on a Sephadex G–150 column (1·8 × 90 cm) pre-equilibrated with 0·01M Tris, pH 8·6, containing 6M urea and 1% mercaptoethanol. It was eluted with the above buffer at a flow rate of 4ml/hr. The first peak was found by SDS–PAGE to contain the A subunit while the second one is the B subunit. Four ml fractions were collected.

TABLE 1. MOLECULAR WEIGHTS OF ABRINS, ABRUS AGGLUTININ AND THEIR SUBUNITS *

Protein	Intact	A subunit	B subunit
Abrin-a	63,000	28,000	35,000
Abrin-b	67,000	32,000	35,000
Abrin-c	63,000	28,000	35,000
Abrin-d	67,000	32,000	35,000
Abrus agglutinin	67,000	32,000	35,000

* Determined by SDS–PAGE.

Electrophoresis on sodium dodecyl sulfate polyacrylamide gel

The purified isotoxins and abrus agglutinin were analyzed by PAGE in the presence of 0·1% SDS. The mol. wts of the isotoxins were similar (Table 1). When abrins were treated with 5% 2–mercaptoethanol at 45°C for 1 hr before electrophoresis, the abrins give rise to one strong and one weak band. The strong band moving slower was the B subunit while the weak band moving faster was the A subunit as designated by OLSNES *et al.* (1974). The molecular size of B subunits of four abrins was similar but that of the A subunit can be divided into two groups, namely, smaller size of abrin-a and -c, and larger size of abrin-b and -d (Fig. 7).

Amino acid composition and carbohydrate determination

The amino acid composition of the four isotoxins and their subunits except those of abrin-d are given in Table 2 and Table 3 respectively. The four isotoxins have similar but not identical amino acid composition. The results of determination of carbohydrate in each subunit are summarized in Table 4. The B chains of abrin-b contain higher amounts of sugar than those of abrin-c or abrin-a, and the B chain has a higher amount of sugar than the A chain does.

Hemagglutination

The isotoxins showed a remarkable difference in their ability to agglutinate human red blood cells. At the concentration of 2 mg/ml, abrin-b does not agglutinate red blood cells, while abrin-a, c and d are able to agglutinate at 1, 200 and 1 μg/ml, respectively. The agglutination caused by the isotoxins can be inhibited by addition of 0·02M D (+)-galactose. But at the concentration of 0·03 μg/ml, abrus agglutinin is able to cause the agglutination of red blood cells.

Lethality

All four purified isotoxins are highly toxic to Swiss white mice by i.p. injection. The LD_{50} values which were determined by the results observed 48hr after injection are 10, 25, 16 and 31μg/kg body wt for abrin-a, b, c and d respectively, and the abrus agglutinin was demonstrated to be non-lethal even at a dose of 1 mg/kg body wt.

TABLE 2. AMINO ACID COMPOSITIONS OF VARIOUS ABRINS AND ABRUS AGGLUTININ*

Amino acid	Abrin-a	Abrin-b	Abrin-c	Abrin-d	Abrus agglutinin	Abrin-A†	Abrin-C†	Abrin‡
Lysine	21·4	21·2	23·1	23·8	26·2	19·1	20·3	12·4
Histidine	8·1	9·2	10·0	8·8	9·3	8·8	6·7	9·7
Arginine	33·0	32·0	32·0	32·9	36·4	34·3	34·9	35·0
Aspartic acid	79·3	73·8	68·2	67·4	75·2	68·1	70·5	58·9
Threonine	45·8	39·8	38·7	38·1	38·3	38·8	46·2	41·6
Serine	51·7	48·0	48·6	48·6	54·0	51·4	61·4	51·2
Glutamic acid	64·0	64·0	64·0	64·0	64·0	56·1	55·9	64·7
Proline	23·0	23·1	24·2	23·1	23·5	18·6	20·7	30·3
Glycine	44·8	40·3	43·5	43·0	37·3	33·6	41·3	34·7
Alanine	34·7	35·2	30·3	37·2	37·4	32·0	32·3	40·5
Half-cystine	ND§	ND	ND	ND	ND	10·0	10·8	4·2
Valine	36·1	32·9	31·2	29·8	35·4	32·0	32·3	33·5
Methionine	10·0	6·0	8·8	7·3	10·2	11·2	12·9	6·7
Isoleucine	34·9	33·2	28·9	30·0	25·5	32·9	33·8	26·4
Leucine	42·4	40·6	39·8	40·0	40·7	43·6	43·3	49·6
Tyrosine	22·4	21·1	21·6	20·8	22·6	22·7	26·9	26·5
Phenylalanine	16·8	16·1	15·5	17·3	18·4	13·5	16·3	23·9
Tryptophan ‖	10·5	11·1	10·5	10·5	12·0	7·9	7·0	—

* Composition in residues per mole for mol. wts shown in Table 1; average of four experiments.
† Reported by WEI *et al.* (1974).
‡ Obtained by summation of A and B chain(OLSNES and PIHL, 1976).
§ ND, not determined.
‖ Determined by spectroscopic method (EDELHOCH, 1967).

TABLE 3. AMINO ACID COMPOSITIONS OF SUBUNITS OF ABRINS AND ABRUS AGGLUTININ*

Amino acid	Abrin-a		Abrin-b		Abrin-c		Agglutinin		Abrin†	
	A	B	A	B	A	B	A	B	A	B
Lysine	4·1	18·8	4·0	18·0	4·1	20·6	7·3	21·5	4·6	7·8
Histidine	3·9	4·0	3·8	5·8	4·2	7·1	4·8	5·2	5·8	3·9
Arginine	19·7	16·0	18·8	15·9	18·4	16·5	20·0	16·4	18·4	16·6
Aspartic acid	30·2	49·4	27·2	48·6	27·0	44·5	28·0	46·7	31·0	27·9
Threonine	18·1	27·0	16·2	23·6	16·8	22·3	18·7	24·0	19·8	21·8
Serine	19·6	30·3	20·0	29·1	21·4	27·5	26·3	29·5	23·4	27·8
Glutamic acid	32·0	32·0	32·0	32·0	32·0	32·0	32·0	32·0	33·3	31·4
Proline	13·7	10·8	10·7	12·9	11·6	12·8	13·4	11·7	14·6	15·7
Glycine	16·3	30·0	15·4	28·6	16·9	28·0	16·2	25·1	16·4	18·3
Alanine	18·3	15·9	18·3	16·3	17·1	13·9	20·6	18·3	17·9	22·6
Half-cystine ‡	1·1	11·2	1·1	8·8	1·1	9·9	1·5	12·4	2·0	2·2
Valine	14·9	19·3	14·4	17·5	14·6	12·8	15·8	20·2	15·0	18·5
Methionine	2·0	8·5	2·1	4·0	1·8	4·7	2·2	7·3	2·6	4·1
Isoleucine	16·0	18·2	15·2	17·8	14·9	13·1	11·4	14·3	13·8	12·6
Leucine	20·2	22·8	19·4	21·6	18·7	20·1	24·2	18·8	22·4	27·2
Tyrosine	8·8	15·7	8·9	11·5	9·9	11·4	9·8	14·0	10·5	16·0
Phenylalanine	10·9	5·8	10·7	5·5	10·6	4·2	11·9	6·8	12·1	11·8
Tryptophan §	3·0	7·8	2·9	7·9	2·8	7·9	2·9	8·4	ND‖	ND

* Composition in residues per mole for mol. wts shown in Table 1; average of four experiments.
† Reported by OLSNES and PIHL (1976).
‡ Determined as carboxymethylcystine (CRESTFIELD *et al.*, 1963).
§ Determined by spectroscopic method (EDELHOCH, 1967).
‖ ND, not determined.

TABLE 4. HEXOSE AND GLUCOSAMINE CONTENTS OF SUBUNITS OF ABRINS AND ABRUS AGGLUTININ

Protein subunits	Hexose (μg/100 μg of subunit)	Glucosamine (No. of residues per mole of subunit)
Abrin-a		
A subunit	0·57	0·8
B subunit	3·90	3·4
Abrin-b		
A subunit	4·12	2·9
B subunit	4·62	4·1
Abrin-c		
A subunit	1·24	0·8
B subunit	3·12	2·3
Abrus agglutinin		
A subunit	3·00	2·1
B subunit	5·68	3·0

DISCUSSION

By using affinity chromatography on Sepharose 4B column, two isotoxins were purified from the jequiriti bean, *Abrus precatorius* (WEI *et al.*, 1974; OLSNES and PIHL, 1976; LIN *et al.*, 1978). By further investigation four isotoxins and one abrus agglutinin can be obtained by Sepharose 4B column chromatography followed by DEAE-cellulose column chromatography and gel filtration.

By using Sephadex G–150 column chromatography in the presence of 6M urea, it was possible to separate the A and B chain; the yield being about 80% for both subunits. The elution profile was contradictory to the expectation that the A subunit eluted before the B subunit has a lower mol. wt than the B subunit. This could be due to the fact that the molecular shape of the B chain is more compact than that of the A chain under our experimental conditions.

Abrin-a, isolated by the present method, corresponds to abrin-C reported by WEI *et al.* (1974) and abrin obtained by OLSNES and PIHL (1976), as judged from the common properties of the toxin: (1) it is absorbed on the Sepharose 4B column and eluted from the column in the presence of galactose; (2) the isotoxin is highly toxic compared to other isotoxins. Abrin-b and abrin-c have properties similar to those reported by WEI *et al.* (1974) as abrin-A and also by OLSNES and PIHL (1976): (1) the isotoxins are retarded on the Sepharose 4B column and are eluted from the column as a peak in the absence of galactose; (2) the isotoxins are less toxic than abrin-a. Abrin-d is newly isolated and not the major toxin present in the jequiriti bean due to its low content and low toxicity. The amino acid composition of abrin-a (Table 2) is very similar to that of abrin-C reported by WEI *et al.* (1974) except the content of glutamic acid, 64 compared to 56 residues per molecule of toxin. There are some differences between the data of A and B chain given by OLSNES and PIHL (1976) and those of abrin-a reported here. Examples are lysine, 12·4 compared to 22·9 residues; aspartic acid, 58·9 compared to 71·5–79·6 residues; half cystine, 4·2 compared to 9·9–12·3 residues. The discrepancy of lysine and half cystine residues is primarily due to differences between the B chains while that of aspartic acid residues is due to differences between both B and A chains (Table 3).

It was reported that most of the carbohydrates in abrins are bound to the B-chain (OLSNES and PIHL, 1976), but as shown in Table 4, all the A chains of the isotoxins and abrus agglutinin contain considerable amount of hexose as well as glucosamine. The differences found may be due to the fact that the seeds used in different laboratories are from different varieties, and these remain to be further investigated.

REFERENCES

BOYD, W. C. (1970) Lectin. *Ann. N. Y. Acad. Sci.* **169**, 168.

CRESTFIELD, A. M., MOORE, S. and STEIN, W. H. (1963) Alkylation and identification of the histidine residues at the active site of ribonuclease. *J. biol. Chem.* **238**, 2413.

EDELHOCH, H. (1967) Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry* **6**, 1948.

GATT, R. and BERMAN, E. R. (1966) Rapid procedure for the estimation of amino sugars on a microscale. *Anal. Biochem.* **15**, 167.

KOSTRABA, N. C., MONTAGNA, R. A. and WANG, T. Y. (1975) Study on the loosely bound non-histone chromatin proteins. *J. biol. Chem.* **250**, 1548.

LIN, J. Y.,TSERNG, K. Y., CHEN, C. C., LIN, L. T. and TUNG, T. C. (1970) New antitumor substance. *Nature. Lond.* **227**, 292.

LIN, J. Y., LEE, T. C. and TUNG, T. C. (1978) Isolation of antitumor protein abrin-a and abrin-b from *Abrus precatorius*. *Int. J. Peptide Protein Res.* **12**, 311.

MCPHERSON, A., JR. and RICH, A. (1973) Studies on crystalline abrin: X-ray diffraction data, molecular weight, carbohydrate content and subunit structure. *FEBS Lett.* **35**, 257.

OLSNES, S. and PIHL, A. (1976) Abrin, ricin, and their associated agglutinin. In: *Receptors and Recognition Series: The Specificity and Action of Animal, Bacterial and Plant Toxins*, Vol. 31, p. 131 (CUATRECASAS, P., Ed.). London: Chapman & Hall.

OLSNES, S., SALTVEDT, E. and PIHL, A. (1974) Isolation and comparison of galactose-binding lectins from *Abrus precatorius* and *Ricinus communis*. *J. biol. Chem.* **249**, 803.

REED, L. J. and MUENCH, H. (1938) A simple method of estimating fifty percent end points. *Am. J. Hyg.* **27**, 493.

ROY, J., SOM, S. and SEN, A. (1976) Isolation, purification and some properties of a lectin and abrin from *Abrus precatorius* Linn. *Arch. Biochem. Biophys.* **174**, 359.

SPACKMAN, D. H., STEIN, W. H. and MOORE, S. (1958) Automatic recording apparatus for use in the chromatography of amino acid. *Anal. Chem.* **30**, 1190.

STAVITSKY, A. B. (1954) Micromethods for the study of proteins and antibodies. *J. Immunol.* **72**, 360.

WEI, C. H., HARTMAN, F. C., PFUNDERER, P. and YANG, W. C. (1974) Purification and characterization of the two major toxic proteins from seeds of *Abrus precatorius*. *J. biol. Chem.* **249**, 3061.

WINZLER, R. J. (1955) Determination of serum glycoproteins. In: *Methods of Biochemical Analysis*, Vol. 2, p. 279 (GLICK, D., Ed.): New York. Interscience Publishers.