Properties of Volkensin, a Toxic Lectin from *Adenia volkensii*

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Volkensin, a highly toxic protein from the roots of *Adenia volkensii* (kilyambiti, kinoria), was purified by affinity chromatography on acid-treated Sepharose 6B. The toxin is a glycoprotein (M₉ 62,000, neutral sugar content 5.74%) consisting of an A subunit (M₉ 29,000) and of a B subunit (M₉ 36,000) linked by disulfide and noncovalent bond(s). The amino acid, amino sugar, and neutral sugar composition of the protein were determined. Volkensin is a galactose-specific lectin and is a potent inhibitor of eukaryotic protein synthesis in whole cells as well as in a cell-free system (a rabbit reticuloocyte lysate). The inhibitory and the lectin activities are functions of the A and B subunits, respectively. Volkensin can be included amongst the ricin-like toxins and resembles most closely modeccin, the toxin of *Adenia digitata*.

A number of plant toxins inhibit protein synthesis by damaging the 60 S ribosomal subunit. This group of toxins includes ricin and abrin, known since the end of last century, and the more recently identified modeccin, from the roots of *Adenia digitata* (Modecca digitata), and viscumin, from the leaves of *Viscum album* (mistletoe) (reviewed by Olsnes and Pihl, 1982). These toxins are all proteins consisting of two subunits: a B subunit, which binds to galactosyl-terminated receptors on the cell membrane, allowing the entry of the A subunit, which inactivates ribosomes in a catalytic manner.

Interest in these toxins is growing for several reasons. Not only are they of interest in their right, but they are also useful models for the study of protein entry into cells, and they are potentially useful as antitumor agents. It was reported that ricin and abrin have antitumour activity (Lin et al., 1970, 1971; Podstata et al., 1977). To obtain drugs with higher selectivity, ricin or its active A chain has been conjugated to antibodies against cell surface markers to obtain "immunotoxins," selectively toxic for cells that are recognized by the antibody (reviewed by Thorpe et al. 1982).

In the present experiments, we have studied the properties of volkensin, a toxin recently purified from the roots of *Adenia volkensii* Harms (kilyambiti, kinoria), a Passifloraceae from Kenya (Barbieri et al., 1984). The results showed that volkensin belongs to the group of plant toxins mentioned above, resembling particularly modeccin, the toxin of another Passifloraceae, *A. digitata* (Refsnes et al., 1977; Stirpe et al., 1978).

**EXPERIMENTAL PROCEDURES**

**Materials**—Roots and seeds of *A. volkensii* were supplied from the University Herbarium, Nairobi, Kenya.

Chemicals for chromatography and electrophoresis, reagents for estimation of protein synthesis, sugars, and protein markers for M₉ determination were obtained from the same sources as in previous work (Barbieri et al., 1986). Na²¹² was obtained from Amersham International, Amersham, Bucks, United Kingdom. Iodogen was purchased from Pierce Biochemistry, Beijerland, Holland.

**Cells**—The Vero cells, HeLa S3 cells, and baby hamster kidney cells used have been cultivated at the Norsk Hydro’s Institute for Cancer Research, Oslo, for years (see Olsnes et al., 1982). The modeccin-resistant variant was isolated from mutagenized Vero cells by growth in medium containing modeccin.

**Purification of Volkensin**—The toxin was extracted from *A. volkensii* roots, precipitated with ammonium sulfate, and purified by affinity chromatography on acid-treated Sepharose 6B (Ersson et al., 1973) as described previously (Barbieri et al., 1984) except that dialysis of the redissolved ammonium sulfate precipitate was replaced by gel filtration on a Sephadex G-25 column. This procedure was more rapid and removed some colored material present in the extract.

Volkensin was also isolated from *A. volkensii* seeds that were ground with an Ultra-Turrax apparatus (Janke & Kunkel, Staufen, West Germany) with 0.14 M NaCl containing 5 mM sodium phosphate buffer, pH 7.2 (8 ml/g of seeds). After overnight stirring at 4 °C, the extract was centrifuged at 20,000 x g for 30 min, and the clear supernatant was applied to a column of acid-treated Sepharose 6B which, after washing, was eluted with 0.2 M galactose in phosphate-buffered NaCl solution.

Throughout all operations, the greatest care was taken to avoid any contact of the body with the roots or with the extract at any stage. This was not only to avoid poisoning, but also because we observed that one person involved in the work developed an allergy to this material.

**Radioiodination**—Volkensin was labeled with ¹²⁵I essentially by the method of Fraker and Speck (1978) as described by Olsnes et al. (1982), except that borate buffer was replaced by a universal buffer (67 mM phosphoric acid, 114 mM boric acid, adjusted to pH 8.5 with NaOH).

**Chemical Determinations**—Electrophoresis was performed on acetic acid strips (Chenevel, Chemetron Chimica, Rozzano, MI, Italy) in 0.35 M alanine/acetate acid buffer, pH 4.5, and in 0.37 M Tris/glycine buffer, pH 9.25. SDS*—polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). The following markers were used for M₉ determinations (all from LKB, Bromma, Sweden), cytochrome c (M₉ 12,300), myoglobin (17,700), carbonic anhydrase (30,000), ovalbumin (45,000), bovine serum albumin (66,250), and bovine serum albumin (57,000 and 78,000).

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* The abbreviations used are: SDS, sodium dodecyl sulfate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

1 G. Olsnes et al., manuscript in preparation.

2 The abbreviations used are: SDS, sodium dodecyl sulfate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
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*M* values were estimated also by gel filtration through a column (80 cm × 1.6 cm) of Bio-Gel P-150, equilibrated with 0.3 M NaCl containing 5 mM sodium phosphate buffer, pH 7.2, eluted at the rate of 12 ml/h, at 4° C. The column was calibrated with the following markers; chymotrypsinogen (*M* < 25,000), ovalbumin, bovine serum albumin (all from Pharmacia Fine Chemicals, Uppsala, Sweden), and glucose 6-P dehydrogenase (104,000) (from Boehringer Mannheim GmbH, Mannheim, West Germany). The isoelectric point and the amino acid, amino sugar, and neutral sugar composition of the toxin were determined as described by Falasca et al. (1982).

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard or spectrophotometrically (Kalb and Bernhauer, 1973).

Cell Culture—Cells were propagated as monolayer cultures in minimum essential medium containing 10% fetal calf serum. The day before the experiments, the cells were seeded into 24-well disposable trays as earlier described (Sandvig and Olsnes, 1982a).

Protein Synthesis—This was measured in a lysate of rabbit reticulocytes or in intact cells from the incorporation of L-[14C]leucine or L-[3H]leucine, respectively, as described by Sargiacomo et al. (1983) or by Sandvig and Olsnes (1982a). Details are given in the legends to the appropriate figures and tables. The ID₅₀ was determined by linear regression analysis. Poly(U)-directed polyphenylalanine synthesis by E. coli ribosomes was measured as described by Grise-Miron et al. (1981).

Hemagglutinating Activity—Blood was collected and the erythrocytes were separated and washed as described previously (Barbieri et al., 1983) and were used either fresh or trypsinized (Lis and Sharon, 1972) and fixed with glutaraldehyde (Turner and Liener, 1975). Hemagglutinating activity was determined in Greiner microtiter plates. Each well contained, in a final volume of 100 µl, 50 µl of a 1% erythrocyte suspension and serial dilutions of the toxin and, when appropriate, 1% mouse albumin (all from Pharmacia Fine Chemicals, Uppsala, Sweden), and glucose 6-P dehydrogenase (104,000) (from Boehringer Mannheim GmbH, Mannheim, West Germany). The isoelectric point and the amino acid, amino sugar, and neutral sugar composition of the toxin were determined as described by Falasca et al. (1982).

RESULTS

Chemical Properties of Volkensin—Volkensin was isolated from the roots of *A. volkensii* by affinity chromatography on acid-treated Sepharose 6B, a procedure commonly used to purify other galactose-specific lectins. The results of a typical preparation are given in Table I. The purified toxin inhibited protein synthesis and agglutinated erythrocytes (see below). After gel filtration on Sephacryl S-200, both activities emerged together in a sharp protein peak (Fig. 1). A single protein peak with apparent *M*₅₂₀₀₀ was obtained also by gel filtration on Bio-Gel P-150 (results not shown). Volkensin was also isolated from the seeds of *A. volkensii* by the same procedure.

When volkensin was analyzed by electrophoresis on cellulose acetate under nondenaturing conditions at pH 4.5 and 5.0, a single major band and a faint minor band were observed. The minor band had a higher mobility at pH 4.5 and a lower mobility at pH 9.25 than the major band (results not shown). On SDS-polyacrylamide gel electrophoresis, volkensin migrated corresponding to a *M*₅₂₀₀₀, and after treatment with 2-mercaptoethanol, two major bands with mobility corresponding to *M*₃₆,₀₀₀ and 29,₀₀₀ and a fainter heavy band with apparent *M*₇₇,₄₀₀ were seen (see Fig. 5 below).

On isoelectric focusing, volkensin showed two intense bands at pH 8.2 and 7.8 and three faint bands at pH 8.5, 8.1, and 7.5 (results not shown).

The amino acid, amino sugar, and neutral sugar contents of volkensin are given in Table I, together with the corresponding values for modeccin. It is clear that the composition of the two toxins is very similar, the main differences being that volkensin contains more half-cystine residues and more than twice as much sugar as modeccin due to a higher content of mannose and galactose. It should be considered, however, that the galactose detected could represent traces of that used to elute the toxins.

The similarities between volkensin and modeccin prompted us to examine whether the two toxins were also immunologically related. When analyzed by immunodiffusion in agarose, volkensin reacted with anti-modeccin serum, giving rise to a single precipitation line which formed a spur with that of modeccin (Fig. 2) as evidence of partial immunological identity.

Inhibition of Protein Synthesis: Cell-free System—Volkensin inhibited protein synthesis by a rabbit reticulocyte lysate, with an ID₅₀ of 5 µg/ml (8.4 × 10⁻⁶ M). The inhibitory effect was abolished when volkensin was heated at 70° C for 20 min, whereas it was greatly enhanced when the toxin was reduced with 2-mercaptoethanol (ID₅₀ = 22 ng/ml (3.7 × 10⁻⁶ M)). The concentration of ribosomes in the assay mixture was 5 × 10⁻⁷ M, as determined from the ribosomes recovered by centrifugation of the lysate and measured as described by Stirpe et al. (1981). Assuming a complete recovery of actively ribosomes, intact and reduced volkensin inactivated ribosomes at a rate of 3 and 357/min, respectively.

Table I

| Preparation                      | Total protein | Specific activity | Total activity | Recovery | Specific activity | Total activity | Recovery |
|----------------------------------|---------------|------------------|----------------|----------|------------------|----------------|----------|
| Crude extract                    | 6,912         | 7.4              | 51,149         |          |                  |                |          |
| Ammonium sulfate precipitate     |               |                  |                |          |                  |                |          |
| Dialyzed                         | 413           | 89.3             | 38,875         | 72       |                  |                |          |
| Volkensin                        | 104           | 199              | 20,676         | 40       |                  |                | 2,048    |

* Determined spectrophotometrically. After dialysis and freeze-drying, 75 mg were recovered.
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FIG. 1. Chromatography of volkensin. Volkensin (300 µg) was applied to a column (83 cm × 1.6 cm) of Sephacryl S-200 previously equilibrated with 0.14 M NaCl containing 5 mM sodium phosphate buffer, pH 7.2. The column was eluted with the same solution, and the Em (c~') of the effluent (2.1-ml fractions) was recorded. The effect of protein synthesis by a rabbit reticulocyte lysate (0-0) and the hemagglutinating activity on trypsinized rabbit erythrocytes (X-X) were determined in the legend to Table I.

The toxin at a concentration up to 120 µg/ml did not affect polyphenylalanine polymerization by E. coli ribosomes (results not shown).

Cells—Volkensin also strongly inhibited protein synthesis by various animal cells (Fig. 3A). In this case, the inhibitory activity was decreased when the toxin was reduced. Thus, the ID50 values for Vero cells were 18 pg/ml (3 × 10^-3 M) and 1.5 ng/ml (2.5 × 10^-6 M) for the native and the reduced toxin, respectively. Even with high concentrations of the toxin, protein synthesis started to decline only after a certain time lag (Fig. 3B). Volkensin inhibited protein synthesis at concentrations 10 times lower than those required for modeccin. Anti-modeccin serum protected cells also against volkensin (Fig. 3C), and this was also the case with NH4Cl or monensin (Fig. 3D), which both protect cells against modeccin (Sandvig and Olsnes, 1982b). Cell mutants resistant to modeccin were equally resistant to volkensin (Fig. 3E). It is clear that volkensin and modeccin act on cells in similar, but not identical, manners.

Hemagglutinating Activity—Volkensin agglutinated human erythrocytes without any specificity for any particular blood group and the erythrocytes of any animal species examined except sheep (Table III). The latter were agglutinated by high concentrations of volkensin if they were pretreated with trypsin, a treatment which greatly enhanced the agglutination of the erythrocytes of all species except horse. In all cases except cat erythrocytes, the hemagglutinating activity of volkensin was consistently twice that of modeccin. In the present experiments, the latter had an activity somewhat higher than that previously observed (Gasperi-Campani et al., 1979).

The effect of various sugars on the hemagglutinating activity of volkensin is shown in Table IV. Only D-galactose and sugars with the same configuration inhibited hemagglutination. The inhibitory sugars had an identical effect on the agglutination of human erythrocytes (group O, Rh') by volkensin and modeccin (results not shown).

Separation of Subunits—On SDS-polyacrylamide gel electrophoresis, volkensin appears constituted by two subunits (see above). On the assumption that they were analogous to the A and B subunits of ricin (Olsnes and Pihl, 1973), their separation was attempted by exploiting the galactose-binding
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**Fig. 3. Effect of volkensin on protein synthesis by cells.** Volkensin and modeccin were added to cells growing in 24-well microtiter plates (5 × 10⁴ cells/well) in minimal essential medium with 10% fetal calf serum. After 18 h at 37 °C, the medium was removed, and leucine-free medium containing 21 mM Hepes buffer, pH 7.7, instead of bicarbonate, 2 µCi/ml ²H]leucine, and no serum was added. After incubation at 37 °C in an atmosphere of air for the time period indicated, the cells were washed twice with 5% (w/v) trichloroacetic acid and dissolved with 0.2 ml of 1 M KOH, and the radioactivity incorporated was measured as described by Sandvig and Olsnes (1982a). A, ability of increasing concentrations of volkensin to inhibit protein synthesis in L-cells (○), Vero cells (●), HeLa S3 cells (▲), and baby hamster kidney cells (△). The incubation with volkensin was for 30 min. B, time course of the effect on protein synthesis of various concentrations of volkensin in Vero cells: 10⁻¹¹ M (●), 10⁻¹⁰ M (▲), and 10⁻⁹ M (▲). C, effect on protein synthesis by Vero cells of volkensin (circles) and modeccin (triangles) alone (solid symbols) or in the presence of anti-modeccin serum (open symbols). The incubation with the toxin was for 30 min. D, ability of NH₄Cl and monensin to protect Vero cells against volkensin: volkensin alone (●), 10 mM NH₄Cl present (○), and 10 µM monensin present (△). E, ability of monensin to inhibit protein synthesis by Vero cells (▲) and by the modeccin-resistant Mod VRes C113 cells (●); the effect of modeccin on the same cells is also shown (● — ●).

capacity of the B subunit. When the reduced toxin was applied to a column of acid-treated Sepharose 6B, only a small amount of protein emerged unretained, with electrophoretic mobility corresponding to that of the smaller subunit. The same protein inhibited cell-free protein synthesis and did not agglutinate erythrocytes (results not shown). The bulk of the reduced toxin applied to the column was retained, could be eluted with galactose, and showed the presence of both subunits (results not shown). When the reduced toxin was applied to a column of untreated Sepharose 4B, two protein peaks were not retained, and a third one was eluted with galactose (Fig. 4). On SDS-polycrylamide gel electrophoresis, peak 1 showed a single band, corresponding to the smaller subunit, peak 2 contained both subunits, the heavier being much more abundant, and peak 3 contained the heavier subunit only (Fig. 5). The lighter subunit had the properties of an A chain and the heavier subunit those of a B chain, as assessed from their effects on protein synthesis and from their hemagglutinating activity (Table V). (The slight inhibitory activity on protein synthesis exerted by both isolated subunits and the slight hemagglutinating activity of the lighter subunit are probably due to contaminating traces of the intact toxin.) The inhibitory activity of the A subunit on cell-free protein synthesis was less than that of the reduced toxin. This was probably due to partial inactivation of the A subunit after separation. Indeed, the inhibitory activity of the A subunit solution was almost completely lost after storage for 2 weeks at 4 °C under sterile conditions in the presence of 1% 2-mercaptoethanol and was not restored by the addition of the B chain (results not shown).

From these results it seems that the two subunits were still linked together after the reduction of volkensin, a finding apparently not consistent with the reduced effect on protein synthesis by cells. A difference between the chromatographies and the experiments with cells was that in the former case the toxin was at a higher concentration and at a lower temperature, which could favor the association of the subunits by forces other than disulfide bond(s). That this was the case was shown by experiments in which reduced and nonreduced volkensin labeled with ¹²⁵I was added to cells. On SDS-
expressed as the lowest concentration causing visible agglutination.

other toxins.

The following sugars did not affect hemagglutination: all, a all, 

Hemagglutination was determined as described in the legend to Table I. Volkensin was added at a final concentration of 2 μg/ml. The following sugars did not affect hemagglutination: D-Alf, L-Alf, D-Alt, D-Ara, 2-deoxy-D-amino-D-Gal, L-Gal, D-Glc, L-Gal, D-Gal, L-Glu, 2-deoxy-2-amino-D-Gal, L-Gal, D-Glc, L-Gal, D-Glu, L-Lyxose, L-Mannose, L-Arabinose, L- Ribose, D-Fucose, L-Fucose, L-Rhamnose, 2-Deoxy-D-galactose, 2-Acetamido-2-deoxy-D-galactose, Methyl-α-D-galactopyranoside, Methyl-β-D-galactopyranoside, Lactose, Melibiose, D-Glactopyranosyl-β-D-thiogalactopyranoside.

polyacrylamide gel electrophoresis, both subunits were visible in the Triton X-100 extract of the cells incubated with the native toxin, whereas the heavier subunit and traces only of the lighter one were present in the cells incubated with reduced volkensin (Fig. 6). This indicates that at low concentration, and high temperature the subunits are separated after reduction with 2-mercaptoethanol, as was the case also with modeccin (Barbieri et al., 1980). The hemagglutinating activity suggests that either the B subunit has two sugar-binding sites, as reported for ricin (Villafranca and Robertus, 1981), or that the toxin may form multivalent aggregates, as observed with viscumin (Olsnes et al., 1982).

Volkensin appears to be similar to the other plant toxins which inhibit protein synthesis, and especially to modeccin. The latter finding is not surprising, since the two toxins are present in taxonomically related plants, both being members of the Passifloraceae. Thus, the two toxins are immunologically related, but there is no evidence of cross-reactivity among them.

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**TABLE III**

 Hemagglutinating activity of volkensin and modeccin

Experimental conditions were as described in the text. Activity is expressed as the lowest concentration causing visible agglutination.

| Erythrocyte species | Untreated erythrocytes | Trypsinized erythrocytes |
|---------------------|------------------------|-------------------------|
|                      | Volkensin               | Modeccin                |
| Human group         |                        |                         |
| A                   | 15.6  1.9              | 31.5  3.9               |
| A1                  | 31.2  1.9              | 62.5  1.9               |
| B                   | 15.6  1.9              | 31.2  1.9               |
| A1B                 | 7.8   1.9               | 15.6  1.9               |
| O                   | 15.6  1.9              | 31.2  1.9               |
| Rabbit              | 15.6  0.47             | 31.2  0.97              |
| Cat                 | 62.5  0.97             | 62.5  0.97              |
| Dog                 | 125  1.9               | 250  3.9                |
| Horse               | 125  125               | 250  250                |
| Ox                  | 125  7.8               | No agglutination*       |
| Sheep               | No agglutination*      | 250  No agglutination*  |
| Rat                 | 250  7.8               | No agglutination*       |

* At concentrations up to 250 μg/ml.

| Sugar               | Minimal concentration inhibiting agglutination (μM) |
|---------------------|--------------------------------------------------|
| D-Galactose         | 18.75                                            |
| L-Mannose           | 31.25                                            |
| L-Lyxose            | 100                                              |
| D-Fucose            | 150                                              |
| L-Arabinose         | 150                                              |
| L-Rhamnose          | 62.5                                             |
| 2-Deoxy-D-galactose | 150                                              |
| 2-Acetamido-2-deoxy-D-galactose | 150       |
| Methyl-α-D-galactopyranoside | 37.5    |
| Methyl-β-D-galactopyranoside | 12.5    |
| Lactose             | 6.25                                             |
| Melibiose           | 25                                               |
| D-Glactopyranosyl-β-D-thiogalactopyranoside | 4.7     |

48 h was 0.32 μg/kg of body weight (95% confidence limits 0.28–0.37) and 1.73 μg/kg (0.78–3.79) for rats and mice, respectively. The delayed LD₅₀ estimated at 14 days was 0.061 μg/kg (0.046–0.083) for rats and 1.38 μg/kg (0.56–3.36) for mice. Thus, volkensin proved to be a very potent toxin, particularly for rats, for which the LD₅₀ was approximately 10-fold lower than that of modeccin (Gasineri-Campani et al., 1978). Rats poisoned with higher doses died between 7 and 12 h after administration. They appeared normal for 1–2 h after poisoning and then they became progressively sedated until death, which occurred suddenly, preceded by short-lasting seizures. The rats which received lower doses and died after several days often showed ascites and wax-like peritoneal fat, a sign of pancreatic lesion.

**DISCUSSION**

Our results demonstrate that volkensin is a toxic protein which inhibits eukaryotic protein synthesis both in cells and in cell-free systems, without affecting protein synthesis by E. coli ribosomes. The toxin is a galactose-specific lectin since it binds to Sepharose and can be eluted with galactose, and (2) has hemagglutinating activity which can be inhibited by galactose and structurally related sugars.

Like ricin and related toxins, volkensin consists of an A and a B subunit, with protein-inhibitory and sugar-binding capacity, respectively. These subunits are disulfide-linked and presumably held together by noncovalent bonds, since they cannot be easily separated after reduction with 2-mercaptoethanol, as was the case also with modeccin (Barbieri et al., 1980). The hemagglutinating activity suggests that either the B subunit has two sugar-binding sites, as reported for ricin (Villafranca and Robertus, 1981), or that the toxin may form multivalent aggregates, as observed with viscumin (Olsnes et al., 1982).
Properties of Volkensin and its subunits. Electrophoresis was performed as described in the text with a 15% polyacrylamide gel. Migration was from top to bottom, and the bromphenol blue front is indicated (BPB). Peak numbers refer to Fig. 4: lane 1, volkensin; lane 2, peak 1 (subunit A); lane 3, peak 2 (subunits A and B); lane 4, peak 3 (subunit B). Markers (all from Sigma) were: bovine serum albumin (M, 68,000), ovalbumin (45,000), carbonic anhydrase (29,000), and lysozyme (14,000).

TABLE V
Properties of the isolated subunits of volkensin

| Protein       | Effect on protein synthesis | Hemagglutinating activity |
|---------------|----------------------------|---------------------------|
|               | Cells                      | Lysate                    | units/mg of protein | units/mg of protein |
| Volkensin     | 2 × 10^8                   | 1571                      |
| Volkensin, reduced | 2 × 10^3                   | 250                       | 40                  |
| Fractions 11–14 (A chain) | 2 × 10^8                   | 222                       | 250                 |
| Fractions 25–29 (A + B chains) | 1 × 10^3                   | Inactive*                 | 500                 |
| Fractions 44–46 (B chain) |                      |                            |                     |

* At 100 ng/ml.

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