

Volkensin from *Adenia volkensii* Harms (kilyambiti plant), a type 2 ribosome-inactivating protein

Gene cloning, expression and characterization of its A-chain

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Volkensin, a type 2 ribosome-inactivating protein from the roots of *Adenia volkensii* Harms (kilyambiti plant) was characterized both at the protein and nucleotide level by direct amino acid sequencing and cloning of the gene encoding the protein. Gene sequence analysis revealed that volkensin is encoded by a 1569-bp ORF (523 amino acid residues) without introns, with an internal linker sequence of 45 bp. Differences in residues present at several sequence positions (reproduced after repeated protein sequence analyses), with respect to the gene sequence, suggest several isoforms for the volkensin A-chain. Based on the crystallographic coordinates of ricin, which shares a high sequence identity with volkensin, a molecular model of volkensin was obtained. The 3D model suggests that the amino acid

residues of the active site of the ricin A-chain are conserved at identical spatial positions, including Ser203, a novel amino acid residue found to be conserved in all known ribosome-inactivating proteins. The sugar binding site 1 of the ricin B-chain is also conserved in the volkensin B-chain, whilst in binding site 2, His246 replaces Tyr248. Native volkensin contains two free cysteinyl residues out of 14 derived from the gene sequence, thus suggesting a further disulphide bridge in the B chain, in addition to the inter- and intrachain disulphide bond pattern common to other type 2 ribosome-inactivating proteins.

Keywords: ribosome-inactivating proteins; cloning; volkensin; kilyambiti plant; adenine polynucleotide glycosylase.

Ribosome-inactivating proteins (RIPs; rRNA N- β -glycosidase; EC 3.2.2.22) are widespread among higher plants, and are also present in an alga [1] (*Laminaria japonica*), a fungus [2] (*Volvariella volvacea*) and bacteria [3] (Shiga and Shiga-like toxins). They are divided into three main groups. Type 1 RIPs are single-chain proteins with N- β -glycosidase activity. Type 2 RIPs are larger proteins consisting of two distinct chains: an A-chain (with the same enzymatic activity as type 1 RIPs) that is linked by a disulfide bridge to a B-chain (which behaves as a lectin, having a binding preference for sugars with the galactose configuration) [4–7]. The third group, type 3 RIPs, includes few representatives, which

occur only in maize and barley and become activated after proteolytic cleavage [6].

Both type 1 RIPs and the A-chain of type 2 RIPs are rRNA N-glycosidases and remove a single adenine from rRNA [3]. It has been reported that RIPs remove adenine from DNA [9] and thus the denomination of ‘polynucleotide:adenine glycosidases’ has been proposed for them. A similar activity has also been found in animal tissues [10]. Here we adopt the recently proposed name of adenine polynucleotide glycosylases [11] (APG), in analogy with the EC nomenclature on nucleic acid glycosylases.

The lectinic B-chain preferentially binds to galactosyl-terminated glycoproteins on the surface of most cells, thus allowing and facilitating A-chain entry into the cell. The A-chain damages ribosomes, and possibly nuclear DNA [12], thus inhibiting protein synthesis and killing the cell. Type 2 RIPs include some potent toxins, such as ricin and abrin from *Ricinus communis* and *Abrus precatorius* seeds, respectively, that have been known for more than a century, and others that have been isolated more recently, i.e. mistletoe lectin I (viscumin) from *Viscum album* [13], modeccin [14,15] and volkensin [16], from the roots of *Adenia digitata* (*Modecca digitata*) and *A. volkensii* Harms (kilyambiti plant), respectively, and a toxic protein from *Abrus pulchellus* seeds [17]. The most potent of all is volkensin, which has a median lethal dose (LD₅₀) for rats of 50–60 ng·kg⁻¹ [18]. In contrast, other type 2 RIPs from *Sambucus nigra* (nigrin b) [19], *S. ebulus* (ebulin 1) [20], *Cinnamomum camphora* (cinnamomin) [21], *Iris hollandica* [22] and *Polygonatum multiflorum* [23] have low toxicity, despite sharing with toxins the same structure of lectinic and

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Abbreviations: APG, adenine polynucleotide glycosylases; CNBr, cyanogen bromide; CTAB, 2% cetyltrimethyl-ammonium bromide; Gdn.HCl, guanidinium chloride; IC₅₀, 50% inhibitory concentration; IPTG, isopropyl thio- β -D-galactopyranoside; LD₅₀, median lethal dose; PVDF, poly(vinylidene difluoride); RIP, ribosome-inactivating protein; VK, volkensin.

Enzymes: rRNA N- β -glycosidase (EC 3.2.2.22).

Note: nucleotide sequence data are available in the EMBL database under the accession number AJ537497.

Note: abbreviations Y, N, R, W, H for wobble code positions follow the IUB code.

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enzymatic chains. For nigrin b, this difference was accounted for, at least in part, by the different intracellular routing of the protein, which was largely excreted by cells [24]. Furthermore, unlike ricin, volkensin is retrogradely transported in the central nervous system [25].

Although these special properties of volkensin deserve attention, they have yet to be extensively investigated. This is mainly because of the difficulties in purifying adequate amounts of protein, and isolating A- and B-chains, as well as the lack of knowledge of volkensin at the gene level. We addressed this at both the protein and the gene level. Here we report the amino acid sequencing of large regions of volkensin A-chain and B-chain, and the cloning of the gene coding for the protein. The DNA segment encoding the A-chain has been expressed in *Escherichia coli* and characterized. Based on the surprisingly high sequence identity between volkensin and ricin, a structure of the protein obtained through homology modelling is proposed and discussed. These findings will lead to detailed investigations of structure-function relationships in the mechanism of action of volkensin.

Materials and methods

Proteins

Volkensin was prepared, as described previously by Stirpe *et al.* [18], from *A. volkensii* roots obtained from a local gardener in Reggio Emilia, Italy. The protein was freeze-dried and stored at -25°C until used. Volkensin B-chain was prepared by affinity chromatography on Blue Sepharose, according to Montanaro *et al.* [26].

Molecular weight determinations

Volkensin, dissolved in $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{CH}_3\text{COOH}$ (47 : 50 : 3, v/v/v), was injected directly into a Platform single-quadrupole mass spectrometer (MicroMass, Manchester, UK). Data were acquired between 600 and 1800 m/z^{-1} at 10 s per scan, using a capillary voltage of 3.6 kV and a cone voltage of 40 V. To determine the relative molecular mass (M_r) of A- and B-chains, they were analysed as a mixture in the same instrumental conditions after full reduction of disulfides by β -mercaptoethanol. Approximately 100 μg of whole protein was reduced for 1 h at room temperature in 100 μL of 10 mM Tris/HCl, pH 7.5, containing 0.5% β -mercaptoethanol. The sample was diluted with 30 μL of trifluoroacetic acid and 30 μL of acetic acid and directly infused into the spectrometer through a glass capillary.

Amino acid sequencing

Volkensin (30 μg) was reduced with 33 mM (final concentration) of 1,4-dithiothreitol in 0.5 M Tris/HCl containing 10% SDS. After incubation for 30 min at 25°C , iodoacetamide was added to a final concentration of 100 mM and incubation continued for 5 min in the dark. A- and B-chains, separated by SDS/PAGE [27], were transferred to a poly(vinylidene difluoride) (PVDF) membrane (Applied Biosystems) and directly subjected to Edman

degradation on a Procise Model 491 sequencer (Applied Biosystems) for N-terminal sequencing [28].

For internal amino acid sequence determination, volkensin (300 μg) was treated in the same way. However, protein transfer was incomplete and most of the A- and B-chains remained on the gel. Therefore, peptides for internal sequence determination were obtained from the A- and B-chains both by in-gel tryptic digestion [29] and cyanogen bromide (CNBr) cleavage (on PVDF membranes) of corresponding bands [30]. Peptides, released from *in situ* digestions, were extracted and separated by RP-HPLC on a Model 1100 system (Hewlett-Packard) equipped with a Phenomenex Jupiter C18 column ($0.46 \times 25\text{ cm}$; 5 μm particle size) or a Vydac C4 column ($0.46 \times 25\text{ cm}$; 5 μm particle size) for tryptic and CNBr peptides, respectively.

CNBr fragmentation of the volkensin B-chain was performed in solution according to Gross [31]. Endoprotease Asp-N (Boehringer GmbH, Mannheim, Germany) was used to cleave native, dimeric volkensin in solution, according to the manufacturer's instructions. All chemicals and enzymes used for digestions and sequence analysis were of analytical grade. Sequence analyses were performed as described previously [28].

Isolation of genomic DNA

Approximately 1 g of *A. volkensii* leaves was frozen and ground to powder in liquid nitrogen. Eight millilitres of extraction buffer [2% cetyltrimethyl-ammonium bromide (CTAB); 200 mM Tris/HCl, pH 8.0; 20 mM EDTA; 1.4 M NaCl; 2% β -mercaptoethanol] were added, and the mixture was incubated for 30 min at 60°C . The extract was re-extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1, v/v/v), and then with chloroform alone. After precipitation of DNA, 10 μg of RNase A was added, and the mixture was incubated for 15 min at 37°C . Genomic DNA was further purified using a Qiagen tube-20 (Qiagen), following the manufacturer's instructions.

Determination of free sulfhydryl groups

Determination of volkensin free sulfhydryl groups was carried out according to Ellman [32]. The protein (1 mg) was dissolved in 1.0 mL of 50 mM Tris/HCl, pH 8.0, containing 5 mM EDTA and 6 M Gdn.HCl, and centrifuged. The protein concentration was then determined spectrophotometrically, using a theoretical $\epsilon_{280\text{nm}}$ value of $1.8\text{ mm}^{-1}\cdot\text{cm}^{-1}$. Two protein aliquots, each corresponding to 150 μg of protein ($\approx 2.5\text{ nmols}$), were used to carry out two independent determinations.

Oligodeoxynucleotide primers

Degenerate primer pools, representing all possible coding sequences of the N-terminal volkensin A-chain (GINUP) and C-terminal volkensin B-chain (GINLOW), were designed and synthesized according to the amino acid sequences of volkensin A- and B-chains. The GINUP primer, spanning positions 1–8 of the volkensin A-chain, was: 5'-GTNTTYCCNAARGTNCNTTYGA-3', while the GINLOW primer, spanning positions 251–258 of the

volkensin B-chain, was: 5'-ARRAACCAITGYTGRT TNGWWTAA-3'.

Two additional PCR-degenerate primers were designed and synthesized (Gibco BRL), based on the internal amino acid sequence of the volkensin A-chain. Both primers were located in regions of low degeneration. The primer pools V144–151 (5'-CARAAYAAAYCARATHGCNYT-3') and V210–217 (5'-ACNGGYTGRAANGCNCRTT RAA-3') corresponded to the volkensin A-chain amino acid sequences 144–151 and 210–217, respectively.

PCR experiments

The degenerate GINUP and GINLOW primer pools, described above, were used in the PCR on the *A. volkensii* genomic DNA template. A typical reaction mixture included: each primer pool (400 µM); DNA (25 ng); dNTPs (200 µM each, Boehringer Mannheim); Amplitaq® DNA polymerase (1.25 U; Perkin Elmer) in 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂; in a total volume of 25 µL. Cycling parameters were: denaturation for 30 s at 95 °C, annealing for 30 s (at various temperatures), and primer extension for 2 min at 72 °C. The reaction was carried out for 35 cycles using a Thermal Cycler System (ThermoHybaid). The PCR mixture was analysed on a 1% agarose gel in 1 × Tris/acetate/EDTA (TAE buffer) and visualized by ethidium bromide staining.

Preparation of the DIG-labelled probe and Southern blot

The internal fragment of the volkensin A-chain, obtained by PCR amplification with V144–151 and V210–217 primer pools, was extracted from the agarose gel using the Qiaex II gel Extraction kit (Qiagen) and labelled with the DIG DNA Labelling kit (Boehringer Mannheim), according to the manufacturer's instructions. The amplification products obtained with the GINUP/GINLOW primer pools were separated on a 1% agarose gel and blotted overnight onto positively charged nylon membrane (Boehringer Mannheim). Hybridization was carried out overnight at 68 °C in Boehringer Standard buffers. Washes and detection were performed according to the manufacturer's instructions.

Cloning and sequencing

The 219- and 1500 bp fragments (obtained by PCR amplification) that corresponded, respectively, to the probe and to the volkensin coding sequence, were isolated from the agarose gel using the Qiaex II gel Extraction kit (Qiagen) and ligated into the pGEM-T easy cloning vector (Promega Biotech). The ligation mixtures were used to transform *E. coli* XL1 Blue (Stratagene) electrocompetent cells using an *E. coli* Pulser (Bio-Rad), according to the manufacturer's manual. Transformed clones were screened by PCR with V144–151 and V210–217 primer pools and the positive clones were sequenced using the AmpliCycle Sequencing kit (Perkin Elmer), according to the manufacturer's instructions. The nucleotide sequence of the volkensin gene has been submitted to the European Molecular Biology Laboratory (EMBL) under the accession number AJ537497.

Volkensin A-chain subcloning and expression in *E. coli*

For heterologous expression of the recombinant volkensin A-chain, the coding sequence was obtained by specific PCR on the full-length volkensin gene. Linker-extended primers were designed to generate a DNA molecule with an *Nde*I site at the 5' end of the A-chain sequence (primer rVKA1) and a stop codon after the last codon followed by a 3'-*Eco*RI site (primer rVKA2). Sequences of the synthetic oligonucleotides were: 5'-ACTCATATGGTTTTCCCAA GGTCCCGTTC-3' for the primer rVKA1 and 5'-TGAGA ATTCTTACCTTGGAGGTTGAGAGCAGACG-3' for the primer rVKA2 (the restriction sites *Nde*I and *Eco*RI and the stop codon are in bold and in italics, respectively). The amplified DNA was recovered from a 1% agarose gel and digested with *Nde*I and *Eco*RI. The resulting DNA fragment was then subcloned into the pET 21a(+) vector (Novagen, Madison, WI) and digested with the same endonucleases. The ligated DNA was transformed by electroporation into competent *E. coli* BL21 (DE3), according to Sambrook *et al.* [33], and the positive transformants were selected by nested PCR. A positive clone, pET 21arVKA, was sequenced to confirm its identity.

To express the recombinant volkensin A-chain, 500 mL of Luria–Bertani broth containing 100 µg·mL⁻¹ ampicillin (LB_{amp}) was inoculated into a 2 L shake flask containing 5 mL of a stationary-phase preculture of *E. coli* BL21/pET 21arVKA. The cultures were shaken at 37 °C and growth was monitored at 600 nm. At an attenuation of 0.2–0.6 at 600 nm (*A*₆₀₀) gene expression was induced by adding isopropyl thio-β-D-galactopyranoside (IPTG). To set up optimal conditions, the expression was carried out at temperatures of 25 °C, 30 °C and 37 °C, and at different induction times (1.5 h, 2 h, 3 h and overnight). The maximum amount of expressed recombinant volkensin A-chain was obtained following incubation at 30 °C for 3 h. We also compared the levels of recombinant volkensin A-chain expression with different concentrations of IPTG. At IPTG concentrations of 0.1–1000 µM, optimal recombinant volkensin A-chain expression was obtained at 50 µM IPTG. After 3 h of induction at 30 °C, cells were harvested by centrifugation at 3000 *g* for 5 min at 4 °C in a J2550 rotor (Beckman centrifuge Avanti J-25), yielding 4–5 g of cells per L of culture. The cells were suspended in 30 mL of lysis buffer (50 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, pH 8.0) and incubated at 20 °C with lysozyme to a final concentration of 200 µg·mL⁻¹ for 30 min. The lysed bacteria were then sonicated (five pulses of 1 min each at a high output setting). Insoluble cell debris and inclusion bodies were separated from soluble components by centrifugation at 20 000 *g* for 1 h at 4 °C. Proteins of both soluble and insoluble fractions were analysed by 12% SDS/PAGE and stained with Coomassie Brilliant Blue.

Isolation and folding of recombinant volkensin A-chain from insoluble inclusion bodies

The sediment from the transformed *E. coli* sonicate was washed twice with 20 mL of STET buffer [50 mM Tris/HCl, 8% (w/v) sucrose, 50 mM EDTA, 1.5% (v/v) Triton-X-100, pH 7.4], according to Babbitt *et al.* [34], to remove

E. coli proteins. The remaining sediment was dissolved in 20 mL of denaturing buffer (6 M Gdn.HCl, 100 mM dithiothreitol, 50 mM Tris/HCl, pH 8.0) by shaking for 16 h at room temperature. Refolding was achieved by dialysis against 50 mM Tris/HCl, 5 mM EDTA, 100 mM dithiothreitol, pH 8.0, containing decreasing concentrations of the denaturing agent (from 4 M to 0 M Gdn.HCl). Any precipitate was separated by centrifugation at 20 000 *g* for 30 min at 4 °C, and an aliquot of the soluble fraction ($\approx 2 \mu\text{g}$) was analysed by SDS/PAGE (Fig. 4, lane 8).

Biological assays

The RNA N-glycosidase activity of recombinant volkensin A-chain was determined on yeast ribosomes, as described previously [35]. Protein synthesis inhibition was determined using a rabbit reticulocyte lysate, as described previously [36].

Alignment of type 2 RIP sequences

A search for sequence similarities was performed with the BLAST program available on-line (<http://www.ncbi.nlm.nih.gov/BLAST>).

Sequences submitted in the SWISS-PROT database were aligned using the CLUSTALW software in the default set-up. The alignment was then analysed using BOXSHADE software.

Molecular modelling of volkensin A- and B-chains

A model for volkensin A- and B-chains was constructed using the SWISS-MODEL system [37] for comparative protein modelling. Crystallographically derived coordinates of the ricin A- and B-chains (PDB entry code 2AAI) refined to 2.5 Å resolution, were used as a template structure [38]. A manual improvement of sequence alignment was carried out using the Swiss-PDB Viewer software, and the resulting SPDBV project files were resubmitted to SWISS-MODEL in 'Optimize Mode'.

Results

Physico-chemical properties of volkensin

Native volkensin, analysed by SDS/PAGE, showed a single band with an M_r of 60 000. In the presence of the reducing agent β -mercaptoethanol, the single band corresponding to the A-chain (M_r 29 000) was separated from the B-chain, which appeared as a doublet with M_r values of 37 400 and 36 000, respectively. By ESI-MS, the observed relative M_r values were (a) $59\,352.45 \pm 7.7$, $59\,505.50 \pm 5.8$ and $59\,625.93 \pm 8.9$ for native volkensin, (b) $28\,064.30 \pm 3.5$ for the reduced A-chain, and (c) $31\,104.74 \pm 3.4$, $31\,266.23 \pm 1.46$, $31\,431.89 \pm 2.6$ and $31\,541.43 \pm 4.5$ for the reduced B-chain. Therefore, the results of ESI-MS experiments revealed that native volkensin occurs in multiple forms, with differences in M_r , which could be a result of microheterogeneity and/or differences in glycation of the B-chain [17], as also shown by its behaviour as a doublet on SDS/PAGE. It should be noted that the mass value obtained for the A-chain is in good agreement with that calculated from the gene sequence (see below). Determination of free sulfhydryl groups on the native volkensin,

performed under denaturing conditions, gave a value of $1.81 \text{ mol}\cdot\text{mol}^{-1}$ of protein.

Determination of the amino acid sequence

Determination of the amino acid sequence of the A- and B-chains of volkensin has been hampered by difficulties in obtaining pure and adequate amounts of separated A- and B-chains, although several separation methods have been attempted under various conditions. This was a result of the insolubility of the A- and B-chains, once reduced, and to low recovery of peptides after fragmentation. Yet, by the strategy and the methodology described in the Materials and methods, > 90% of the amino acid sequence of the A-chain, and $\approx 40\%$ of that of B-chain, were determined and are tabulated in Table 1. It is of interest that for sequence positions 101–123 of the A-chain, four peptide forms were found. Following Asp-N digestion, H or R were found at position 105, and N or H at position 118. These findings were confirmed when we analysed the CNBr peptides pCN-1 (with R-105) and pCN-3 (with N-118), and tryptic peptides TP-5 (with H-105) and TP-6 (with N-118). The form with H-118 was not found in the tryptic peptides.

Isolation and cloning of the volkensin gene

On the basis of the N- and C-terminal amino acid sequences of volkensin A- and B-chains, obtained by Edman degradation, two degenerate oligonucleotide pools were synthesized and used as primers for the PCR amplification of *A. volkensii* genomic DNA. When the specificity of the PCR reaction was optimized by gradually increasing the annealing temperature using gradient PCR, at 58 °C only one product was obtained with the size (1.5 kbp) expected on the basis of the volkensin M_r (data not shown).

To exclude that the amplification had generated a non-specific band, we performed PCR on genomic DNA using two new degenerate oligonucleotide pools (V144–151 and V210–217), and thus obtained a 219 bp volkensin-specific fragment. This was used directly as a hybridization probe in a Southern blot analysis on PCR products obtained with the primers based on N- and C-terminal amino acid sequences. The single hybridizing band of the expected size (1.5 kbp, data not shown) was eluted from the agarose gel and subjected to a nested PCR using the same primers as used for amplification of the 219 bp volkensin probe; a 219 bp fragment was obtained, which corresponded exactly to the size of the probe used as a positive control. Finally, to confirm its identity, the 1.5 kbp fragment was subcloned and sequenced as described in the Materials and methods.

The DNA sequence analysis revealed that volkensin is encoded by a 1569-bp ORF without introns. The gene (see Fig. 1) contains an internal linker sequence of 45 bp, which links the 750 bp coding sequence of the A-chain (250 amino acid residues with a calculated M_r of 28 071.04) with the 774 bp sequence encoding the B-chain (258 amino acid residues with a calculated M_r of 28 483.23). The A-chain contains two cysteinyl residues at positions 156 and 245, as also found in ricin D (Cys171 and Cys259). The B-chain contains 12 cysteinyl residues (at positions 4, 20, 39, 59, 63, 78, 149, 162, 188, 191, 195 and 206), three more than found in the ricin B-chain, and two potential glycosylation sites at

Table 1. Amino acid sequences of S-modified volkensin A- and B-chains and of cyanogen bromide (CN; p, precipitate; s, soluble), tryptic (TP) and endoproteinase Asp-N (AN) peptides, used to assemble the amino acid sequences of the two chains. The amino acid residues found to be present in four forms of the Asp-N peptide 101–123 are shown in bold.

Peptide/protein	Sequence	Sequence position	Notes
A-chain			
S-modified	VFPKVPFDVPKATVESYTRFIRVLRDELGG	1 → 31	N-terminal
pCN-1	DVRNAYLLGYLSHNVLYHFNDVSASSIASVFPDAQRRQL	69 → 107	
pCN-3	RNYAPERDQIDHGIVELAYAVDRLYYSQNNNQIALGLVI	117 → 155	
pCN-2	VAEASRFRIEGLVRQSIVGLGEYRTFRPDAL	160 → 191	
pCN-4	YSIVTQWQTLSERIQGSFNGAFQPVQLGYA	193 → 222	
sCN-5	LFVCSQPPR	242 → 250	
TP-1	VFPK	1 → 4	N-terminal
TP-2	VFPDVPK	5 → 11	
TP-3	VFPKVPFDVPKATVESY	1 → 17	N-terminal
TP-4	VLRDELGGVSPQGR	23 → 38	
TP-5	HQLPFGGGYPSMR	105 → 117	
TP-6	NYAPERDQIDHGIVELAYAVDR	118 → 139	
TP-7	DQIDHGIVELAYAVDR	123 → 139	
TP-8	YIEGLVR	168 → 174	
TP-9	TFRPDALMYSIVTQWQTLSE	185 → 205	
TP-10	IQGSFNGAFQPVQLGYASDPFYWDNVAQAI	206 → 235	
TP-11	ASDPFYWDNVAQAIR	222 → 237	
TP-12	LSMLFVCSQPPR	238 → 250	C-terminal
AN-1	VFPKVPFD	1 → 8	N-terminal
AN-2a	DAQRHQLPFGGGYPSMRNYAPER	101 → 123	
AN-2b	DAQRHQLPFGGGYPSMRHYAPER	101 → 123	
AN-2c	DAQRRQLPFGGGYPSMRNYAPER	101 → 123	
AN-2d	DAQRRQLPFGGGYPSMRHYAPER	101 → 123	
AN-3	DHGIELAYAV	127 → 137	
B-chain			
S-modified	DPVCPSGETT	1 → 10	N-terminal
CB-1	DPVCPSGETTAFIVGRDGR	1 → 20	N-terminal
CB-2	WPCKSSQANQLWTL	37 → 51	
CB-3	RSQSTLSKCLACSGSC	180 → 195	
CB-4	DVKESNPSLNEIIAHPWHGNSNQWFL	232 → 258	C-terminal
TP-1	DPVCPSGETTAFIVGRDGR	1 → 19	
TP-2	WTLKRDGTIR	49 → 58	
TP-3	WEVWDNGTIINPASGR	88 → 103	
TP-4	WTLYADGTIR	171 → 180	
AN-1	NEIIAHPWHGNSNQWFL	241 → 258	C-terminal

positions 93 and 133 (Fig. 1). Two main differences were found between the gene sequence and the sequence of the A-chain. We found Pro instead of Leu, and Asp instead of Glu, at positions 180 and 182, respectively. These differences were consistently found in several amplification experiments or peptide-digestion patterns. The amino acid residues found in the gene sequence at positions 105 and 118 were R and N, respectively.

Sequence comparison between volkensin and other type 2 RIPs

A multiple alignment of the volkensin sequence with other type 2 RIPs is shown in Fig. 2, while in Fig. 3, identity/similarity matrices of the A- and B-chain amino acid sequences are reported. The two highest percentages of identity were found between the A-chain of volkensin and those of ricin D (34.8%) and cinnamomin (33.2%), isolated from the seeds of *R. communis* and *C. camphora*, respect-

ively. All other identities were lower (ranging from 29.2 to 30.5%), even though the amino acid residues reported to be implicated in the active site of RIPs were conserved within the sequence of the volkensin A-chain. In contrast, the volkensin B-chain showed a higher degree of identity when compared with other type 2 B-chains. Identity values were between 43.5% (nigrin b and ebulin) and 49.6% (ricin D).

Expression, folding and biological activity of the recombinant volkensin A-chain

The 750 bp DNA fragment encoding the volkensin A-chain was isolated by PCR, cloned into the pET-21a expression vector and expressed in the *E. coli* host strain, BL21 (DE3). As shown in Fig. 4 (lane 5), a band of ≈ 29 kDa was present in the induced clones against the background of total protein. Densitometric scanning indicated that it represented more than 70% of total proteins (data not shown). Most of the recombinant volkensin A-chain, identified by direct

gttttccccagggtccggttcgacgtcccaaggccacggtagagctacactcggttt 60
 V F P K V P F D V P K A T V E S Y T R F 20
 ataagggtttaagggaacagctggcggtgtgtttctccgaggggaatacgcaggctg 120
 I R V L R D E L A G G V S P Q G I R R L 40
 aggaatccggttgagattcagccttcacagggtttttatttatacaactcaccggttac 180
 R N P A E I Q P S Q G F I L I Q L T G Y 60
 gtaggctccgctcacttgataatggacgtccgcaatgcgtatctattgggttatctgagc 240
 V G S V T L I M D V R N A Y L L G Y L S 80
 cacaacgttctgtatcaactcacaagatgtctctgcctcttcgatccgagtggtttccca 300
 H N V L Y H F N D V S A S S I A S V F P 100
 gacgcccacgtcgacagttgccatttgaggcggtatccacagcatcgaaactatgcg 360
 D A Q R R Q L P F G G G Y P S M R N Y A 120
 ccggagagagatcaaatggacatggaatogtgcgaactggcagcgtgttgataggctc 420
 P E R D Q I D H G I V E L A Y A V D R L 140
 tactatagtcacaaacacaaatcagatgcgctgggactogttatctgcgcgggaatgggt 480
 Y Y S Q N N N Q I A L G L V I C A G M V 160
 gcagaagcatcccggttccgctacatcgaggcggtgtgtgcgcaaaagcatttgtgggcca 540
 A E A S R F R Y I E G L V R Q S I V G P 180
 ggagactacgcaactttcagacgggtagctgtatgtactcaactcgtgacacagtgccag 600
 G D Y R T F R P D A L M Y S I V T Q W Q 200
 actctttcagaaagaatccagggaatccttcaacggagcttttcagccagttcagctgggg 660
 T L S E R I Q G S F N G A F Q P V Q L G 220
 tatgcccagatccctttttatgggacacagctgcacaggccatccaggctgttaactc 720
 Y A S D P F Y W D N V A Q A I T R L S L 240
 atgctatctgtctgtctcacaacctccaaggcaatccgattcccccctgtgtgaaggctct 780
 M L F V C S Q P P R Q S D S P L V I R S 260
 ttgttggtataggaacagatctctgtctgccttccggggagacgactcggttcaactcgtggg 840
 F V D R N D P V C P S G E T T A F I V G 280
 cgggacggcggtgtgtggaagtggaagtggaattcttcgacggcaacaaagtagcag 900
 R D G R C V D V K V E E F D G N K V Q 300
 atgtggcggtgcgaactcagacacaaatgcgaacagctgtggaactcgaagagagagcggc 960
 M W P C K S S Q N A N Q L W T L K R D 320
 actattcgtgtccaggggaagtgctgactgtgaggagcccgcaactgtacgctatgatc 1020
 T I R C Q G K C L T V R S P Q L Y A M I 340
 tgggactgcacactttttatgtctccgcacacaaagtggaagtggtgggacacgggaacc 1080
 W D C T T F Y A P A T K W E V W D N G T 360
 atcatcaaccccgctccgggaggtgtgtgacccgcccactggggagggcggtcacc 1140
 I I N P A S G R V L T A P T G E A G V T 380
 ctcaacttgcaattcaacgagtagcgcctagcaggcggtggagggtgacaaatgtgaca 1200
 L N L Q F N E Y A A S Q A W R V T N V T 400
 gtaoctacgggtgacaacacttgttgatgatgatctctgcctggaaacacacgggaac 1260
 V P T V T T I V G D E T L C L E T N G N 420
 ggtgtatgtgtggccaactcgtggaaggcgaacagagatggagcgtgtatgcg 1320
 G V W L A N C V K G K A Q Q R W T L Y A 440
 gatggcaccatcgcctccagctccaccctcagcaaatgtctcgcctgcagtgctcctgc 1380
 D G T I R S S T L S K C L A C S G S C 460
 gtcaagttggcaagatcgtcaacacggactgtgtggaatccgccaacagcogttgtgtac 1440
 V K L A K I V N T D C A G S A N S R W Y 480
 ctgcacaaactacggcggtcgtggaatctcgtacggcgaatgtgtgaggaatgaaagag 1500
 F D N Y G G I V N L R T G M V M D V K E 500
 tccaaatccgagtcacaaagaaataatgcaccacogtggaatggaaactccacacgcaa 1560
 S N P S L N E I I A H P W H G N S N Q Q 520
 tggttccta 1569
 W F L 523

Fig. 1. Full-length sequence and derived amino acid sequence of the volkensin gene. Proteolytic cleavage giving mature A- and B-chains results in excision of the linker peptide (indicated by an arrow). Potential N-glycosylation sites are underlined.

N-terminal sequencing via Edman degradation of the 29 000 Da molecular mass band (data not shown), appeared to be sequestered in the inclusion body fraction (Fig. 4, lane 7). After renaturation, as described in the Materials and methods, the recombinant volkensin A-chain was homogeneous upon SDS/PAGE analysis (12% gel; Fig. 4, lane 8). The molecular mass of recombinant volkensin A-chain was estimated to be $\approx 29\,000$ Da. Approximately 5 mg of recombinant volkensin A-chain was obtained from 1 L of induced *E. coli* culture.

Biological activity of the recombinant A-chain of volkensin

When recombinant volkensin A-chain was tested for enzymic N-glycosidase activity on yeast ribosomes, the diagnostic Endo fragment [39], released from the 26S rRNA after aniline treatment, was detected (data not shown). Furthermore, recombinant volkensin A-chain was found to inhibit protein synthesis in a rabbit reticulocyte lysate, with

a 50% inhibitory concentration (IC_{50}) of 79.3 ng mL^{-1} , slightly higher than that of the native A-chain of volkensin ($IC_{50} = 22\text{ ng mL}^{-1}$), as reported previously [18].

Molecular modelling of the volkensin A- and B-chains

Owing to the relatively high sequence identity/similarity between volkensin and ricin, the crystallographically derived atomic coordinates of the latter were used to perform homology modelling, using the 'optimize mode' of the SWISS-MODEL, as described in the Materials and methods. Figure 5 shows the 3D structures predicted for volkensin A- and B-chains.

Discussion

Volkensin, a type 2 RIP isolated from *A. volkensii* roots, is the most potent plant toxin known. The present work was undertaken with the aims of cloning the volkensin gene and obtaining recombinant A-chain, the enzymically active toxin chain.

We found that the 1.5 kbp gene encoding volkensin is without introns. The absence of introns has been previously reported for other RIPs, such as type 2 RIPs from Iris bulbs [22], PMRIP [23], ricin [40,41], abrin [42,43] and viscumin [44], all homologous to volkensin. A comparison of the volkensin gene sequence with the amino acid sequence of the protein A-chain revealed differences at positions 180 and 182. Furthermore, amino acid sequencing of the A-chain also identified differences in residues at positions 105 and 118. These findings suggest polymorphism and the existence of more than one volkensin coding gene, as in the case of other type 2 RIPs, such as the lectin gene family of *R. communis* [45]. Further experimental work (i.e. Southern blot analysis on genomic DNA) is needed to confirm this hypothesis.

As reported in the literature [4,5], type 2 RIPs share a high degree of identity, with the A-chains showing a lower percentage of identity than the B-chains. This has been explained by the hypothesis that the B-chain coding region is derived from an event of gene duplication [46]. The volkensin B-chain also appears to be organized into two domains (residues 1–136 and 137–258, respectively), with $\approx 26\%$ identity between the two domains.

The gene sequence and previous studies [18] show that volkensin contains a total of 14 cysteinyl residues (the highest number among type 2 RIPs): two in the A-chain and 12 in the B-chain. As revealed by the free sulfhydryl group determination experiment, only two of these cysteinyl residues are in the SH form, while the remaining 12 should form six disulfide bridges. It is known for other type 2 RIPs, such as ricin, that (a) a cysteinyl residue at the C-terminal end of the A-chain forms an interchain disulfide bond with a cysteinyl residue at the N-terminal end of the B-chain, and (b) that the two domains of the B-chain are each organized around a pair of disulfide bridges. Therefore, on the basis of the sequence alignment of Fig. 2 and the 3D model (data not shown), we suggest that this disulfide scheme also holds for volkensin A- and B-chains (i.e. Cys245 of the A-chain is linked to Cys4 of the B-chain; Cys20 to Cys39 and Cys63 to Cys78 for domain 1 of the B-chain; Cys149 to Cys162 and Cys188

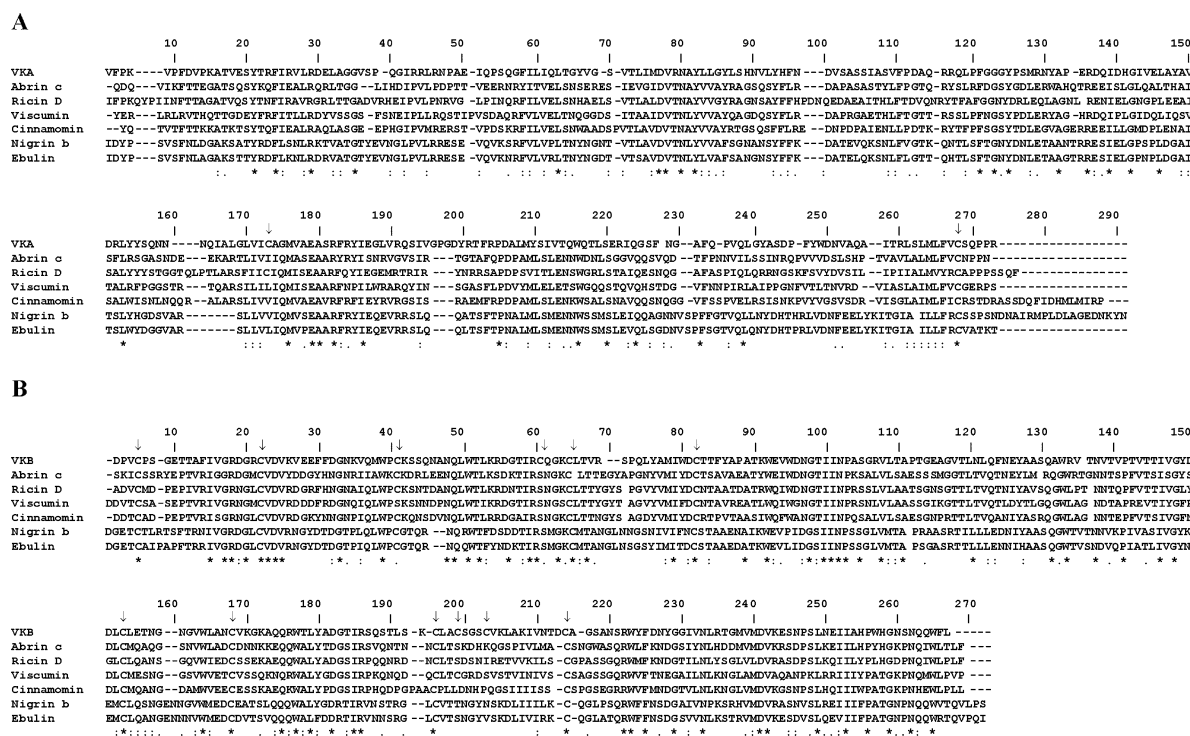


Fig. 2. Multiple alignment of type 2 ribosome-inactivating proteins. A multiple alignment between volkensin, abrin c, ricin D, cinnamomin, viscumin, nigrin b and ebulin is reported for the A-chain (I) and the B-chain (II). The single letter code has been used for the amino acids. Identical residues (*), conserved substitutions (:) and semiconserved substitutions (.) are reported. Arrows indicate the cysteinyl residues.

A	VKA	Abrin c	Ricin D	Viscumin	Cinnamomin	Nigrin b	Ebulin
VKA	---	40.1	45.2	40.5	43.6	40.9	44.1
Abrin c	29.8	---	52.6	53.9	56.2	47.8	50.8
Ricin D	34.8	40.7	---	48.0	50.7	45.1	48.7
Viscumin	30.5	40.9	36.1	---	49.1	43.3	48.6
Cinnamomin	33.2	47.4	41.8	39.2	---	50.5	51.4
Nigrin b	29.2	33.0	31.8	31.4	38.0	---	82.4
Ebulin	30.4	35.7	33.9	36.7	39.1	79.4	---

B	VKB	Abrin c	Ricin D	Viscumin	Cinnamomin	Nigrin b	Ebulin
VKB	---	56.7	57.6	57.0	54.9	53.2	53.5
Abrin c	48.7	---	68.1	61.4	63.4	51.7	53.5
Ricin D	49.6	59.3	---	70.0	72.0	55.8	58.0
Viscumin	46.0	52.7	62.0	---	67.2	52.0	50.9
Cinnamomin	44.7	56.6	64.0	60.4	---	54.2	55.7
Nigrin b	43.5	39.8	43.9	40.5	42.8	---	83.1
Ebulin	43.5	43.5	46.1	40.9	45.0	77.4	---

Fig. 3. Identity/similarity matrix for the comparison of type 2 ribosome-inactivating proteins (RIPs). The identity/similarity matrix of seven type 2 RIP A-chains (A) and B-chains (B) is shown. Identity values are reported below the diagonal axis and represent the percentage of identical amino acid residues. Similarity values are listed above the diagonal axis.

to Cys206 for domain 2 of the B-chain). Of the remaining four cysteines (i.e. Cys156 of the A-chain, and Cys59, Cys191 and Cys195 of the B-chain) two should be in the reduced form while the other two should form a disulfide bond. On the basis of the 3D model, it is reasonable to assume that the free cysteines are Cys156 of the A-chain and Cys59 of the B-chain, as the first is buried inside the structure and quite distant from any other cysteinyl residue, while the second appears to be isolated on domain 1 of the B-chain (data not shown). Conversely, the last two cysteines, 191 and 195, are placed in a loop region in domain 2, hence sufficiently close to each other to allow the formation of a disulfide linkage.

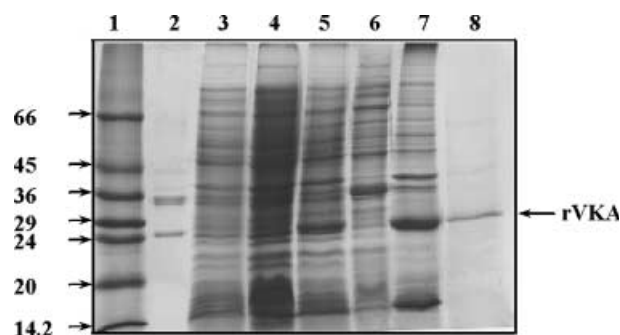


Fig. 4. Expression and purification of recombinant volkensin A-chain in *Escherichia coli* BL21(DE3). SDS/PAGE was performed under reducing conditions and the gel was stained with Coomassie Brilliant Blue. Lane 1, molecular weight markers; lane 2, plant-derived volkensin; lane 3, total proteins in the lysate of non-transformed BL21(DE3); lanes 4 and 5, total fractions before and after induction, respectively; lane 6, soluble fraction after induction; lane 7, cell sediment showing the recombinant volkensin A-chain inclusion body fraction; lane 8, refolded, soluble recombinant volkensin A-chain.

The catalytic key residues involved in the enzymatic mechanism of the ricin A-chain are all conserved in volkensin. These include Tyr80 (Tyr74 for volkensin: hereafter, numbering in parenthesis refers to the volkensin sequence), Tyr123 (113), Glu177 (162), Arg180 (165) and Trp211 (199). These residues are located at equivalent positions in the 3D structure of both proteins (see inserts I and II of Fig. 5A). On the other hand, residues located near

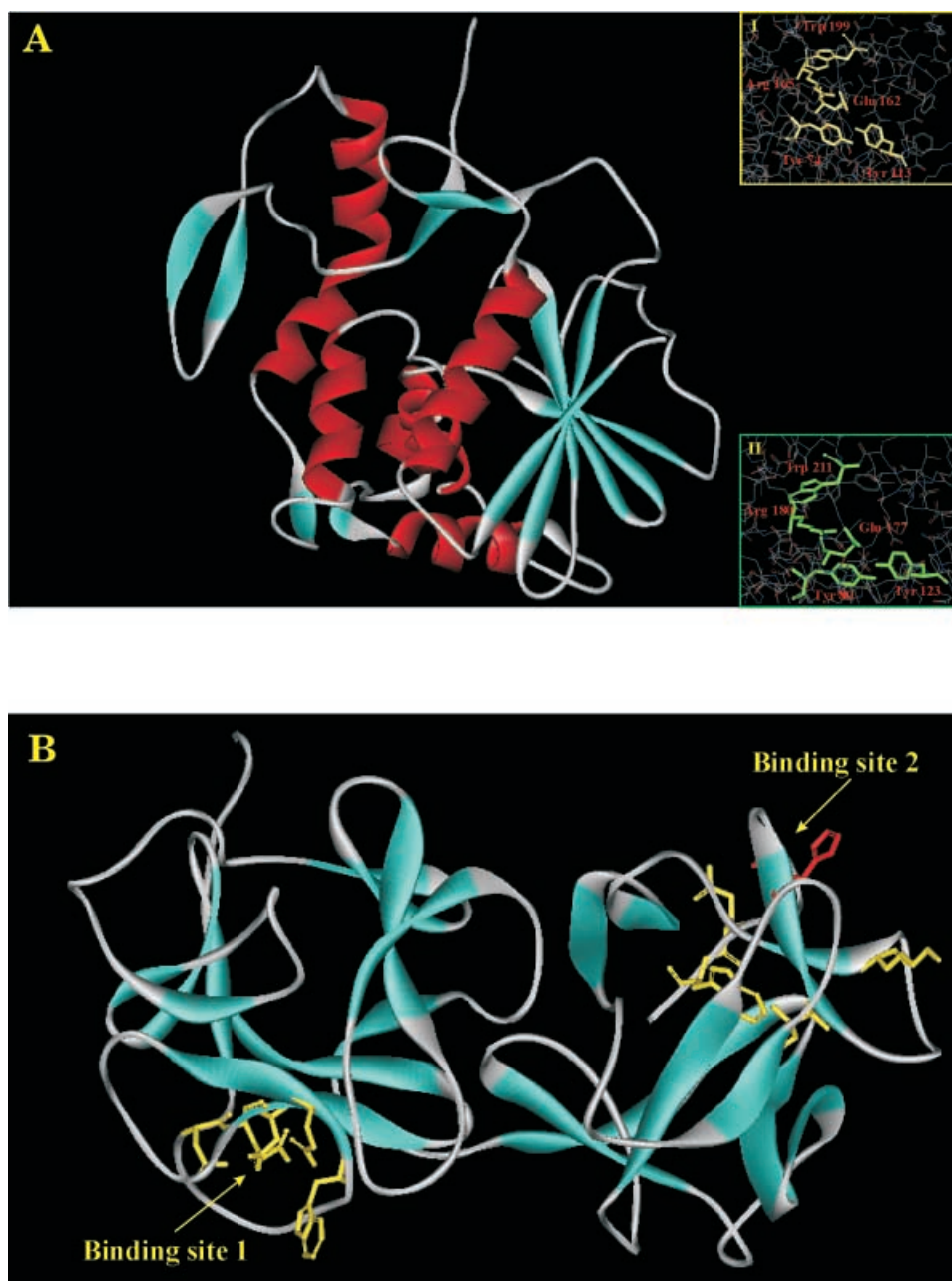


Fig. 5. 3D-models of volkensin A-chain (A) and B-chain (B). The inserts of (A) show the amino acid residues of the active site of volkensin (I) and ricin (II), respectively. Binding sites 1 and 2 of the volkensin B-chain are also indicated, with His246 shown in red. Strands of β -sheets are represented in green, while α -helices are in red.

the active site and thought to contribute to the protein stability are only in part conserved. Some [Asn78 (72), Arg134 (123), Ala178 (163)] also occur in the volkensin A-chain, while Gln173, Glu208 and Asn209 are replaced with Gly158, Val196 and Thr197, respectively. Ser215, located near the active site and highly conserved in both type 1 and 2 RIPs through evolution [47], was also found in the volkensin A-chain (position 203). Tyr21 (17), Phe24 (20) and Arg29 (25) are also conserved at the N-terminus.

Like all type 2 RIP lectin chains, the volkensin B-chain consists of two subdomains comprising short strands of

β -sheets interconnected by turns and loops (Fig. 5B). A more detailed comparison between the carbohydrate-binding site of ricin and the volkensin B-chain indicated that all amino acid residues constituting the binding site 1 of ricin B-chains (Asp22, Gln35, Trp37, Asn46 and Gln47) are fully conserved in the volkensin B-chain. Most amino acid residues (Asp234, Ile246, Asn255, Gln256) of the ricin B-chain binding site 2 are also conserved, except for Tyr248, which is replaced with His246 in the volkensin B-chain (reported in red in Fig. 5B). This replacement was recently found in the sequence of PMRIPm, a type 2 RIP isolated

from the monocot *P. multiflorum* [23], and closely resembles the corresponding sites of the ricin agglutinin A-chain [48] and ricin E from castor bean seeds [49]. Site-directed mutagenesis studies performed on the ricin B-chain have shown that Tyr248 is an essential residue for galactose-binding activity and that its replacement with His246 drastically reduces this activity [50]. In fact, the introduction of an additional positive charge in binding-site 2 prevents the hydrophobic interaction between the pyranose ring of galactose and the aromatic ring of Tyr248. Although this finding suggests that carbohydrate binding site 2 of the volkensin B-chain is weakly functional, further studies on binding affinity and docking of galactose and GalNAc in both binding sites 1 and 2 of the volkensin B-chain are required. However, it should be borne in mind that the replacement of Tyr248 with Phe, an aromatic residue, has also been suggested to lower the toxicity of ebulin 1, as compared with other toxic type 2 RIPs [20].

Interestingly, in spite of the high degree of conservation of amino acid residues involved both in the A-chain active sites and in the B-chain carbohydrate-binding sites of type 2 RIPs, there are remarkable differences between volkensin and other type 2 RIPs in terms of general toxicity, the ability to selectively destroy some cellular types [51–53], and in the retrograde transport along neurons [25], all properties that make volkensin a useful tool in neurological research. As the association of the A- and B-chains may be of relevance for toxicity and appears to be mediated by hydrophobic forces [54], further investigations on the conservation of polar and hydrophobic interactions occurring at chain interfaces should clarify the structure–function relationships responsible for the different activities of this potent toxin. Furthermore, knowledge of the differences in the amino acid sequence between volkensin and ricin may allow us to eliminate the differences in mutants and to identify the residues responsible for the higher toxicity of volkensin.

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