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Cloning and expression of the B chain of volkensin, type 2 ribosome inactivating protein from *Adenia volkensii* harms: Co-folding with the A chain for heterodimer reconstitution

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Abstract

Type 2 ribosome inactivating proteins (RIPs) include some potent plant toxins, among which ricin from *Ricinus communis* and abrin from *Abrus precatorius* seeds, have been known for more than a century. Two other type 2 RIPs belong to this class of proteins, both isolated from plants of the same family (Passifloraceae), modeccin and volkensin, from *Adenia digitata* and *Adenia volkensii* roots, respectively. Volkensin is probably the most potent plant toxin known, with an LD_{50} for rats of 50–60 ng/kg. Here we report the cloning, expression and renaturation of recombinant volkensin B chain. Furthermore, starting from separately expressed A and B chains, a co-association procedure was set-up, leading to *in vitro* heterodimeric volkensin reconstitution. The recombinant heterodimer was characterized by N-terminal sequence analysis and its hemagglutinating activity assessed. In parallel, we have explored the carbohydrate-binding properties of native volkensin with the aim to correlate toxin-specific properties (i.e., axonal transport along neurons) to lectin's sugar-binding preferences.
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Volkensin, the most toxic type 2 ribosome inactivating protein (RIP)[1] isolated from *Adenia volkensii* roots [1], is a 60 kDa glycoprotein consisting of two disulfide-linked subunits. The lectinic B chain preferentially binds to galactosyl-terminated glycoproteins on the surface of most cells, thus allowing and facilitating the entry of the enzymically active A chain into the cell [2]. After internalization and translocation of the toxin to cytosol, the catalytic chain inactivates the 60S eukaryotic ribosomal subunit, thereby inhibiting protein synthesis and causing cell death. The gene encoding pre-provolkensin has been cloned [3]. Despite the high sequence identity and structural similarity, remarkable biological differences have been found among type 2 RIPs. Different central nervous system (CNS) cell populations can display different susceptibility to the toxic insult by volkensin and ricin [4]. This finding could be ascribed to differences in the metabolism of different cell populations or to the intracellular sorting of the toxins depending upon which receptor molecule on the cell surface they bind to [4]. An additional application of RIPs, especially type 2, derives from their peculiar property: the axon transport. Ricin and all related toxins (abrin, modeccin, viscumin, and volkensin), once injected into peripheral nerves, can be retrogradely transported along the axon to the neurons, which are killed (suicide transport) [5–9]. However, when toxins are injected in the CNS, only modeccin and volkensin, but not ricin and abrin, are transported to other areas through the projecting neurons [10,11].

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[1] *Abbreviations used:* RIP, ribosome inactivating proteins; CNS, central nervous system; rVKA and rVKB, recombinant volkensin A and B chain, respectively; rV, recombinant heterodimeric volkensin.

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Moreover, once internalized, volkensin is found in Golgi bodies (Stirpe, personal communication). Thus, modeccin and volkensin are useful tools to study neural connections in the CNS and to cause neuronal damage resembling those of Alzheimer's disease.

In this regard, adequate amounts of the lectin in homogeneous form are required to understand: (i) the molecular mechanism involved in the binding of volkensin to cell glycoreceptors, (ii) the reasons for its high toxicity, (iii) the influence of glycosylation in the structure–function relationships, and (iv) the pathway responsible of its retrograde transport along neurons.

Volkensin A and B chains are difficult to purify from the native volkensin to the degree of purity required for the study of their biological properties. Furthermore, engineered forms of dimeric volkensin could be produced to set up biological experiments. Here we report cloning, expression and renaturation of recombinant volkensin B chain (rVKB). Furthermore, a preliminary co-association procedure was set-up, leading to *in vitro* volkensin heterodimer reconstitution from separately expressed A and B chains. The recombinant heterodimer was characterized by N-terminal sequence analysis and by assessing its hemoagglutinating activity.

Materials and methods

Materials

Restriction endonucleases and T4 DNA ligase were purchased from Boehinger Mannheim GmbH (Mannheim, Germany). Plasmid purification kit and that for the elution of DNA fragments from agarose gel were obtained from Qiagen (Milan, Italy). Expression vector pET22b(+) was from Novagen (Madison, WI) and *Escherichia coli* strain BL21(DE3) were from AMS Biotechnology (Lugano, Switzerland). Plasmid pGEM-Teasy and other reagents for DNA manipulation were from Promega Biotech (Milan, Italy).

DNA manipulation, transformation and plasmid purification were performed according to previously published methods [12]. Native volkensin was prepared from *A. volkensii* roots as described by Stirpe et al. [13] and recombinant volkensin A chain as reported [3].

Methods

Volkensin B-chain subcloning

For heterologous expression of the recombinant rVKB, the coding sequence was obtained by specific PCR on the full-length volkensin gene as previously described [3]. 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 rVKB1) and a stop codon after the last codon followed by a 3′ *Xho*I site (primer rVKB2). Sequences of the synthetic oligonucleotides were: 5′-GAC **TCATAT**GGATCCTGTCTGCCCTTCCG-3′ for the primer rVKB1 and 5′-TGA**CTCGAG** *TTA*TAGGAACC ATTGCTGGTTGG-3′ for the primer rVKB2 (the restriction sites *Nde*I and *Xho*I and the stop codon are in bold and in italics, respectively). The resulting product was purified by agarose gel electrophoresis and subcloned in pGEM-Teasy vector (Promega). The coding region of volkensin B chain was then subcloned as *Nde*I–*Xho*I fragment into the expression vector pET22b (Novagen, Madison, WI) and transformed by electroporation into strain *E. coli* BL21 strain BL21(DE3) for heterologous expression. The positive transformants were selected by nested PCR and a positive clone, pET22b/rVKB, was sequenced to confirm its identity.

Expression in Escherichia coli

The pET 22b/rVKB plasmid was transformed by electroporation into *E. coli* BL21(DE3) for heterologous expression. For rVKB expression, 500 mL of LB_{Amp} medium were inoculated in a 2 L shake flask with 5 mL of a stationary grown pre-culture of *E. coli* BL21 clone containing the pET 22b/rVKB plasmid. The growth of shaken cultures at 37 °C was monitored at 600 nm. At 0.2–0.6 OD_{600nm}, gene expression was induced by adding IPTG. To optimize rVKB expression conditions, different temperatures (20, 30, and 37 °C) and induction times (2, 4 and 16 h) were tested. Recombinant protein production was estimated on total *E. coli* lysate by densitometric analysis. The maximum amount of expressed rVKB after induction was obtained at 37 °C for 4 h. The levels of rVKB expression with different IPTG concentrations were also compared. Over a range of IPTG concentrations between 5 and 1000 μM, optimal rVKB expression was obtained at 50 μM IPTG. After 4 h of induction at 37 °C, cells were harvested by centrifugation at 3000*g* at 4 °C for 5 min in a J2550 rotor (Beckman centrifuge Avanti J-25). Cells were suspended in 30 mL lysis buffer (50 mM Tris–Cl, 100 mM NaCl, 2 mM EDTA, 5 mM DTT, and 1 mM PMSF, pH 8.0) and incubated at 20 °C with lysozyme (final concentration 100 μg/mL) for 30 min. Lysed bacteria were then sonicated with 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 Blue Coomassie R-350.

Isolation of rVKB from insoluble inclusion bodies

The sediment from the transformed *E. coli*, after sonication, was washed four time with 20 mL of STET buffer (50 mM Tris–Cl, 8% (w/v) sucrose; 5 mM DTT, 50 mM EDTA, 1.5% (v/v) Triton X-100, pH 7.4), according to Babbit et al. [14], to remove *E. coli* proteins. Two additional washes were carried out with the same buffer without Triton X-100. The remaining sediment was dissolved in 20 mL of denaturing buffer (6 M Gdn–Cl, 100 mM DTT, and 50 mM Tris–Cl, pH 8.0) by shaking for 16 h at room temperature. Each expression step was monitored by 12% SDS–PAGE analyses performed as described [15].

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