Isolation, characterization and molecular cloning of AnMIP, a new α-type phospholipase A$_2$ myotoxin inhibitor from the plasma of the snake *Atropoides nummifer* (Viperidae: Crotalinae)

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Abstract

A new phospholipase A$_2$ (PLA$_2$)-inhibitory protein was isolated from the plasma of *Atropoides nummifer*, a crotaline snake from Central America. This inhibitor was named AnMIP, given its ability to neutralize the activity of basic PLA$_2$ myotoxins of its own and related venoms. The cDNA of AnMIP was cloned and sequenced, showing that it belongs to the α group of phospholipase A$_2$ inhibitors (PLIs). AnMIP appears as a homotrimer in the native state, held together by non-covalent forces, with a subunit molecular mass of 22,247–22,301 and an isoelectric point of 4.1–4.7. This trimeric structure is the first observed in a PLIα from American crotaline snakes, previously reported only in Asian species. Sequencing, mass spectrometry, and analytical isoelectrofocusing indicated the existence of isoforms, as reported for other PLIαs isolated from snake plasma. The inhibitory profile of AnMIP showed specificity towards group II PLA$_2$s, either belonging to the catalytically-active (D49) or -inactive (K49) subtypes, exemplified in this study by *Bothrops asper* myotoxin I and *A. nummifer* myotoxin II, respectively. By phylogenetic analysis it was shown that AnMIP is closely related to CgMIP-II, previously isolated from the plasma of *Cerrophidion godmani*, showing 93% amino acid sequence identity.
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Keywords: Atropoides nummifer; Phospholipase A$_2$; Myotoxin; Inhibitor; PLI-alpha; Snake venom

1. Introduction

Phospholipases A$_2$ (PLA$_2$s) [E.C. 3.1.1.4] catalyze the Ca^{2+}-dependent hydrolysis of the *sn*-2 ester linkage of glicerophospholipids, yielding fatty acids and lysophospholipids. Based on their primary structure and disulfide bond pattern, PLA$_2$s have been classified into eleven groups (Six and Dennis, 2000). Secretory PLA$_2$s present in snake venoms depend on a conserved catalytic network and a water molecule for their enzymatic activity (Berg et al., 2001). Snake venom PLA$_2$s can induce a variety of pharmacological effects by mechanisms that are either dependent or independent of their catalytic activity, such as neurotoxicity, myotoxicity, anticoagulant effect, platelet aggregation or inhibition, hemolytic, hypotensive and edema-inducing activity (Kini, 2003). Variants of PLA$_2$s in which D49 is substituted, mainly by K49, have been described in Viperidae snake venoms. Despite lacking phospholipolytic activity, these K49 PLA$_2$ homologues display *in vivo* and *in vitro* pharmacological activities associated with their ability to interact with and to destabilize biological membranes (Lomonte et al., 2003).

The natural resistance of snakes to the toxic components of their own venoms, or to those of other species, has been attributed to several mechanisms, including the presence of neutralizing protein factors in their blood plasma (Fortes-Dias et al., 1994; Perales et al., 1995; Faure, 2000). Toxin-inhibitory proteins have also been found in the blood of mammals (Pérez and Sánchez, 1999; Rocha et al., 2002). The PLA$_2$ inhibitors (PLIs) or myotoxin-inhibitory proteins (MIPs) isolated from snake plasma are proteins that inhibit the enzymatic and/or toxic activities induced by venom PLA$_2$s. Based on their

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primary structures, these inhibitors have been classified into three groups, namely α, β, and γ (Ohkura et al., 1997). The structural determinants that define each group include: a region homologous to the carbohydrate recognition domain (CRD) of C-type (Ca^{2+}-dependent) lectins, in PLIαs; the presence of nine leucine-rich tandem repeats, flanked by cysteine and proline-rich clusters, for PLIβs; and two repeats of a cystein pattern characteristic of the three-finger motifs, in PLIγs (Lizano et al., 2003).

PLIαs are oligomers, each subunit consisting of 147 residues, with an N-glycosylation site at N103, four half-cystines at positions 64–141 and 119–133, and molecular masses ranging from 18 to 25 kDa (Lizano et al., 2003). PLIαs have been isolated from Trimeresurus flavoviridis (Kogaki et al., 1989), Agkistrodon blomhoffii siniticus (Ohkura et al., 1993), Bothrops asper (Lizano et al., 1997), Cerrophidion godmani (Lizano et al., 2000), and Bothrops moojeni (Soares et al., 2003). In addition, a homologous protein, but lacking inhibitory activity, has been isolated from the non-venomous snake Elaphe quadrivirgata (Okumura et al., 2003).

The CRD-like domain of PLIαs presents homology with other members of the C-type lectin-like domain (CTLD) family (Drickamer, 1999), including proteins that are able to interact with PLA₂s, such as the pulmonary surfactant apoprotein A (Fisher et al., 1994), the M-type PLA₂ receptor (Lambeau and Lazdunski, 1999) and its soluble form (Higashino et al., 2002). PLIα binding to PLA₂ is independent of calcium (Kogaki et al., 1989; Okumura et al., 2005), and of PLI glycosylation (Nobuhisa et al., 1998; Soares et al., 2003).

By partial deletion analysis, a positive interaction between T. flavoviridis PLIα and acidic PLA₂s was mapped to the CTLD region, and this binding capacity was further restricted to the C-terminal 136–147 residues (Nobuhisa et al., 1998). In this region, two hydrophobic tripeptides (Nobuhisa et al., 1998) and the residue Y144 (Okumura et al., 2005) were shown to be involved in the interaction. Based on chimeras between A. blomhoffii siniticus PLIα and E. quadrivirgata PLI-like protein, as well as site-directed mutagenesis studies, Okumura et al. (2005) proposed that PLIαs form a trimeric structure, in which the variable amino-terminal region positioned previous to the conserved CTLD is responsible for trimerization, and subsequent formation of a central pore, which would be the structure responsible for PLA₂ binding. According to this model, variations in the region 13–36 would contribute to the specificity of the inhibitor towards distinct PLA₂s, whereas residues located near the central pore, including those in the C-terminal region, would directly interact with the PLA₂ (Okumura et al., 2005).

Atropoides nummifer is a crotaline snake distributed along the Central American isthmus, commonly known as "jumping viper" or "mano de piedra" (Solórzano, 1989). Two basic K49 PLA₂s homologues, myotoxins I (Gutiérrez et al., 1986) and II (Angulo et al., 2000), have been isolated from its venom. In this work we report the isolation, characterization and molecular cloning of a new myotoxin-inhibitory protein, AnMIP, present in the blood plasma of this species.

2. Materials and methods

2.1. Purification of PLA₂ myotoxins

Crude venoms of A. nummifer and B. asper, respectively, were pools obtained from at least twenty specimens collected in Costa Rica and kept at the serpentarium of the Instituto Clodomiro Picado, University of Costa Rica. A. nummifer myotoxins I (Gutiérrez et al., 1986) and II (Angulo et al., 2000), as well as B. asper myotoxin I (Gutiérrez et al., 1984), were purified as originally described, by cation-exchange chromatography on carboxymethyl-Sephadex C-25 (Pharmacia). Homogeneity of the toxins was assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE; Laemmli, 1970) and by reverse-phase high performance liquid chromatography (RP-HPLC) on a C4 column (250×4.6 mm; Vydac), eluted at 1.0 mL/min with a gradient from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid (TFA). Purified taipoxin, a group I PLA₂ from the venom of Oxyuranus scutellatus (Fohlman et al., 1976), was kindly donated by Dr Ivan Kaiser, University of Wyoming. Bee (Apis mellifera) PLA₂ (Shipolini et al., 1971), a group III PLA₂, was purified from crude venom (New Techniques Laboratory) by RP-HPLC on a C8 column (250×10 mm; Vydac), eluted at 2.0 mL/min with a gradient from 0 to 60% acetonitrile in 0.1% TFA.

2.2. Purification of AnMIP

An affinity matrix was prepared by coupling a mixture of purified A. nummifer myotoxins I and II (12 mg of each) to an NHS-activated HiTrap HP column (5 mL), following the manufacturer's instructions (Amersham Biosciences). For the purification of AnMIP, blood was collected from three adult A. nummifer specimens using 0.38% (w/v) sodium citrate as anticoagulant, and the plasma was separated by centrifugation and stored at −20 °C. Plasma aliquots of 2 mL were diluted 1:5 in PB buffer (0.1 M sodium phosphate, pH 7.2), and recirculated through the affinity column during 2 h at a flow rate of 1 mL/min. After extensive washing of the column with PB buffer until the eluent absorbance at 280 nm returned to baseline, the bound protein fraction was eluted with 0.1 M glycine-HCl, pH 2.8, and collected in tubes containing 0.5 M Tris–HCl, pH 8.8. This fraction was finally concentrated by ultrafiltration under nitrogen pressure, using PBS (0.04 M sodium phosphate, 0.12 M NaCl, pH 7.2) and a size-exclusion membrane with a molecular mass cutoff of 10 kDa (Amicon). Protein concentration was determined by the micro-BCA method (Pierce) using bovine serum albumin (BSA) as a standard. Homogeneity was assessed by SDS-PAGE and RP-HPLC. This myotoxin-binding protein, named AnMIP, was aliquoted and stored at −20 °C.

2.3. Biochemical and immunochemical characterization of AnMIP

The subunit composition and molecular mass of AnMIP were estimated by SDS-PAGE under reducing (2-mercaptoethanol,

5% v/v) and non-reducing conditions (Laemmli, 1970), followed by Coomassie blue R-250 staining. A set of molecular mass markers (Pharmacia) was included as a reference.

The molecular mass of the oligomeric protein in its native state was estimated by analytical gel filtration chromatography on a Superdex 200 HR 10/30 column, using an FPLC system (Pharmacia Biotech) with PBS pH 7.2 as eluent, at a flow rate of 0.4 mL/min. A calibration curve was constructed by running standard proteins (Pharmacia Biotech) under identical conditions. K_{av} was calculated by the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is elution volume, V_0 is void volume, and V_t is total column volume. Analyses were performed in triplicate.

Chemical cross-linking was utilized to assess the number of subunits composing the oligomeric protein. AnMIP was incubated for 2 h at 37 °C in the presence of increasing concentrations of the homobifunctional agent bis(sulfosuccinimidyl) suberate (BS^3, Sigma), in 50 mM HEPES buffer, pH 7.5 (Staros, 1982). Then, the different samples were analyzed by SDS-PAGE under reducing conditions, as described above.

The molecular mass of AnMIP was determined by electrospray ionization (ESI)-mass spectrometry on a LCQ^{Duo} Finnigan ion trap instrument equipped with a nano-electrospray ionization source, as described by Barona et al. (2006). The amino-terminal sequence of AnMIP was determined by direct Edman degradation on PVDF-adsorbed protein, on an Applied Biosystems 494 amino acid sequencer, operated according to the manufacturer's instructions.

The isoelectric point of AnMIP was determined by analytical isoelectrofocusing in polyacrylamide gels containing carrier ampholytes in the pH range 2.5–6.5 (Pharmacia Biotech) on a mini-IEF cell (Bio-Rad Laboratories) at 100 V for 15 min, followed by step increases to 200 V for 15 min and 450 V for 60 min. Protein markers of known pI values (Pharmacia) were included as a reference.

Glycosylation of AnMIP was determined by dot-blotting, as described by Keren et al. (1986). Three microgram of purified AnMIP, *B. asper* myotoxin I (negative control), or *A. mellifera* PLA$_2$ (positive control), respectively, were adsorbed onto a nitrocellulose membrane strip. Excess binding sites on the membrane were saturated with 2% BSA-PBS for 30 min. After washing with 0.2% BSA-PBS, oxidation of carbohydrates was performed by adding 1% periodic acid-PBS for 1 h. The solution was washed thoroughly, and the resulting aldehydes were reacted with a peroxidase-hydrazide conjugate (52 mU/mL) (Sigma) in 0.2% BSA-PBS for an additional hour. After washing the unbound conjugate, color was developed with H$_2$O$_2$/4-chloro-1-naphtol substrate.

Antigenic cross-reactivity between AnMIP and the previously characterized BaMIP (Lizano et al., 1997) was assessed by enzyme-immunoassay. Wells of microtiter plates were coated overnight with 0.2 μg of either AnMIP or BaMIP, diluted in 100 μl of 0.1 M Tris, 0.15 M NaCl, pH 9.0. After washing with FALK buffer (0.05 M Tris, 0.15 M NaCl, 20 μM ZnCl$_2$, 1 mM MgCl$_2$, pH 7.4) wells were blocked with 100 μl of 1% BSA-FALK for 30 min. After decanting, 100 μl of rabbit anti-BaMIP serum (1:1500) (Lizano et al., 1997), or a normal rabbit serum control, were incubated for 1 h. After washing, 100 μl of a goat anti-rabbit immunoglobulins-alkaline phosphatase conjugate (1:2000) were incubated for an additional hour. Color was developed with *p*-nitrophenylphosphate and recorded at 410 nm on a Multiskan microplate reader (Flow Laboratories). Samples were assayed in triplicate wells.

2.4. Inhibitory activity of AnMIP

In all inhibition experiments, the inhibitor was incubated with the corresponding PLA$_2$ or PLA$_2$-homologue toxin for 30 min at room temperature, in PBS. Molar ratios were calculated on the basis of reported molecular mass values of each PLA$_2$, the subunit mass of AnMIP obtained by mass spectrometry, and the oligomeric state information obtained by chemical cross-linking studies. Analyses were performed in duplicate.

PLA$_2$ activity was determined using egg yolk phospholipids as substrate, followed by extraction and titration of the released fatty acids as described by Dole (1956). Enzymatic activity was also assessed by an indirect hemolysis assay in agarose gels containing egg yolk phospholipids (Gutiérrez et al., 1988). PLA$_2$s belonging to groups I (taipoxin, 1 μg), II (*B. asper* myotoxin I, 10 μg), and III (bee venom PLA$_2$, 0.25 μg) were tested. The enzyme amounts selected correspond to the linear regions of previously established concentration-activity calibration curves.

Myotoxic activity was evaluated *in vitro*, by quantifying the cytolysis of confluently growing myotubes differentiated from the murine myoblast cell line C2C12 (ATCC CRL-1772), as described by Lomonte et al. (1999). In brief, after aspiration of the growth medium, myotoxins alone, or preincubated with AnMIP, were added to the cultures in 96-well plates and incubated at 37 °C. After 3 h, the release of lactate dehydrogenase to supernatants was determined by a kinetic assay (LDH-P mono, Biocon Diagnostik). Controls for 0% and 100% cytolysis consisted of medium alone, and 0.1% Triton X-100, respectively. AnMIP was tested against *A. nummifer* myotoxin II and *B. asper* myotoxin I, using a challenge dose of 20 μg/well.

2.5. cDNA cloning and sequencing

Total RNA was isolated from *A. nummifer* liver with the NucleoSpin RNA L kit (Macherey-Nagel) according to manufacturer's instructions. cDNA synthesis was carried out with the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas Life Sciences), using Oligo(dT)$_{18}$ primers. Primers complementary to the consensus non-coding sequences flanking the reading frames of reported inhibitors were used for the PCR amplification. Primer sequences were as follows: α-F 5'-ACCGCAGAGTTTAAAGATG-3', α-R 5'-GCCGGTC-TTCCTTCT-3'; β-F 5'-GAAAGATACAGGATG-3', β-R 5'-TTCAGGTAACAGGTG-3' (both pairs of primers designed with the Oligo Primer Analysis Sofware 4.04; Wojctech Rychik), and γ-F 5'-ATCCTCACTAAAGAGCCA-3', γ-R 5'-GGATGAAATTGCTGACCT-3', as reported by Lizano et al. (2000). PCR amplification reactions were carried out with the low error-prone *Pfu* DNA polymerase (Fermentas). Cycling conditions were as follows: 4 min at 94 °C, followed by
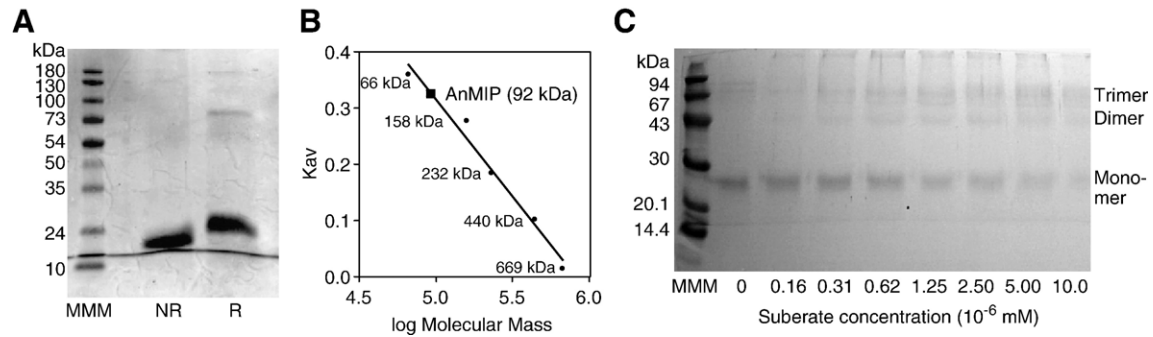
Fig. 1. A. SDS PAGE analysis of AnMIP under non reducing (NR) and reducing (R) conditions. MMM: molecular mass markers (mass indicated to the left). B. Molecular mass determination for native AnMIP (■) by gel filtration chromatography. The molecular masses of the markers (●) utilized for the calibration curve are indicated. K_{av} is defined in Materials and methods. C. Chemical cross linking analysis for AnMIP. The inhibitor was incubated with increasing concentrations (as indicated in the bottom of the gel) of bis(sulfosuccinimidyl)suberate, and then analyzed by SDS PAGE. MMM: molecular mass markers (mass indicated to the left).

30 cycles of 30 s at 94 °C, 1 min at 53, 48 and 50 °C for α, β and γ inhibitors, respectively, and 2 min at 72 °C. Following the last cycle, *Taq* DNA polymerase (Pharmacia Biotech) was added and incubated for 20 min at 72 °C, in order to incorporate poly (A) tails to the blunt-ended PCR products generated by the *Pfu* DNA polymerase. PCR products were analyzed by 1.5% agarose gel electrophoresis in 0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA-Na$_2$, pH 8.0 buffer (TBE), and visualized with ethidium bromide. For the PCR amplification, clones containing the α CgMIP-II (Lizano et al., 2000), β Abs-PLI (Okumura et al., 1998) and γ CgMIP-I (Lizano et al., 2000) were utilized as reaction controls. The reaction products were purified with a DNA extraction kit (Fermentas), following manufacturer's instructions. Ligation to the plasmid pTZ57R/T and transformation of XL1-Blue *Escherichia coli* cells were performed with the InsT/Aclone PCR Product Cloning kit (Fermentas). Plasmid DNA purification was performed with the NucleoSpin Plasmid kit (Macherey-Nagel). Sequencing reactions were carried out with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), and analyzed with an ABI PRISM 310 Genetic Analyzer. Four clones, obtained from two independent retrotranscription and amplification reactions, were sequenced in both senses, with the same primers utilized for the PCR amplification. Manual depuration and analysis of the sequences was performed with the BioEdit Sequence Alignment Editor (Hall, 1999).

2.6. Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment of PLIαs was performed with Clustal W (Thompson et al., 1994). Sequences included in the alignment were the following (when available, NCBI database accession numbers are indicated in square brackets; other sequences were obtained from the literature): *A. nummifer* AnMIP (present study) [DQ657241], *T. flavoviridis* PLIα, subunits A and B (Inoue et al., 1991) [P21755 and P21756, respectively], *Agkistrodon blomhoffii siniticus* PLIα (Okumura et al., 1999) [BAA86973.1], *B. asper* BaMIP (Lizano et al., 1997) [P81077], *C. godmani* CgMIP-II (Lizano et al., 2000), *B. moojeni* BmjMIP (Soares et al., 2003) [JC7936] and *E. quadrivirgata* PLIα-like protein (Okumura et al., 2003) [BAC53925.1]. Phylogenetic

trees were calculated using PROTDIST and NEIGHBOR (Felsenstein, 1995) and drawn with TreeView 1.6.6 (Page, 1996). *Caenorhabditis elegans* hypothetical protein Y48E1B.9 (*C. elegans* Sequencing Consortium, 1998) [Y48E1B.9] was included as an outgroup.

3. Results

3.1. AnMIP purification and characterization

AnMIP was purified from the plasma of *A. nummifer* by affinity chromatography using a mixture of the two basic PLA$_2$ myotoxins present in the venom of this species, which are closely related Lys49 isoforms. From 20 mL of plasma, approximately 1.6 mg of AnMIP was recovered. The inhibitor appeared as a band of approximately 26 kDa under reducing conditions, whereas it migrated as a band of 19 kDa under non-reducing conditions, in SDS-PAGE (Fig. 1A). Using gel filtration chromatography, the molecular mass of the native protein was estimated to be 92 kDa (Fig. 1B). This was in agreement with chemical cross-linking analysis, where BS^3 was able to covalently stabilize a proportion of the AnMIP subunits as dimeric (∼50 kDa) and trimeric (∼80 kDa) structures (Fig. 1C), which did not dissociate under the reducing conditions of SDS-PAGE. No multimeric structures of higher mass were
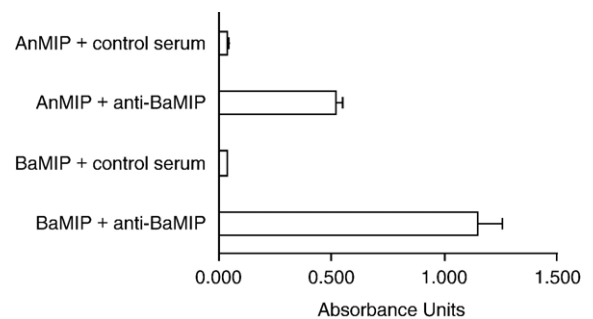

Fig. 2. Cross reactivity between AnMIP and BaMIP evaluated with a rabbit antiserum elicited against BaMIP (anti BaMIP), by enzyme immunoassay. Control serum corresponds to a non immune rabbit. Bars represent mean ± SD of triplicate wells.

detected. Taken together, these determinations suggested AnMIP to be a trimeric protein.

Analytical isoelectrofocusing of AnMIP resolved two bands, corresponding to pI values of 4.1 and 4.7, respectively (not shown). By means of a sensitive hydrazide-peroxidase conjugate assay, AnMIP tested positive for glycosylation. In addition, AnMIP showed a significant antigenic cross-reactivity with BaMIP, an α-type inhibitor (Fig. 2), by enzyme-immunoassay. Antibodies to BaMIP recognized AnMIP with a signal of approximately 45% of that obtained with the homologous antigen (Fig. 2), therefore suggesting AnMIP to belong to the group α inhibitors.

3.2. Inhibitory activities of AnMIP

The ability of AnMIP to inhibit the myotoxic activity of snake venom PLA$_2$s was evaluated in vitro, using cultured myotubes as targets. As shown in Fig. 3, AnMIP was able to reduce by 67% and 93% the toxic activity of A. nummifer myotoxin II and B. asper myotoxin I, respectively, when preincubated at a molar ratio of 1:1. At an AnMIP:toxin molar ratio of 1:3, a 45% reduction in the activity of A. nummifer myotoxin II was obtained, whereas the effect of B. asper myotoxin I remained unchanged (Fig. 3). The limited amounts of purified AnMIP precluded testing its inhibitory action at molar ratios higher than 1:1. Myotube cultures exposed to AnMIP alone, at the higher concentration utilized in inhibition tests, did not present microscopical alterations or increases in LDH activity in comparison to control cultures exposed to medium alone.

Inhibition of PLA$_2$ activity by AnMIP was evaluated on egg yolk phospholipids, either titrimetrically or by an indirect hemolytic test in agarose gels. Given the absence of basic D49 PLA$_2$s in A. nummifer venom, myotoxin I from B. asper, a related crotaline species, was utilized as a representative of group II PLA$_2$s. When preincubated at a molar ratio of 1:1, AnMIP inhibited the enzymatic activity of B. asper myotoxin I by 42%. No inhibition was observed at an AnMIP:toxin molar ratio of 1:3 (not shown). On the other hand, the group I PLA$_2$ taipoxin, and the group III bee venom PLA$_2$ were not inhibited by AnMIP at a molar ratio of 1:1. Increasing the AnMIP: toxin molar ratio to 2:1 did not inhibit the enzymatic activity of taipoxin, whereas a slight decrease of about 25% was observed for the bee venom enzyme (not shown).

3.3. cDNA cloning, sequencing and phylogenetic analysis

Total RNA from A. nummifer liver was isolated in order to generate cDNAs. A 500 bp product was amplified and cloned. Four clones, obtained from two independent retrotranscription reactions, were randomly selected for bidirectional nucleotide sequencing. Two different sequences were obtained, and the nucleotide and deduced amino acid sequences for both variants of AnMIP are presented in Fig. 4. As shown, six nucleotide substitutions were found among these sequences. Three such substitutions implied amino acid changes in the mature coding region, predicting a sequence identity of 97% among both variants. The deduced amino acid sequence presented a 19

amino acids putative signal sequence, typical of secreted proteins. The deduced mature sequences are 147 residues long, presenting the conserved cysteine residues at 64, 119, 133, and 141, and the putative glycosylation site at N103, characteristic of PLIαs. The deduced N-terminal sequence was corroborated by direct amino acid sequencing, up to the 10th residue, showing the sequence DEKDSDVQML.

The deduced amino acid sequences of the two isolated clones predict theoretical molecular masses of 16,378.2 and 16,466.3, respectively. By ESI-MS, two main signals corresponding to mass values of 22,247.0 and 22,301.0 were obtained. The difference between the predicted and the observed molecular mass values would be caused by the glycosylation of the protein.

The AnMIP sequence was aligned with those of other PLIαs, as presented in Fig. 5. From residues 49 to 143 the mature protein presents the CTLD, the most conserved region among these inhibitors. There is a high sequence similarity among inhibitors, except in the case of EqPLI-LP, the homologous non-inhibitory protein from E. quadrivirgata. The C. elegans hypothetical protein Y48E1B.9 (CeCLECT), a homologue of the α group PLIs, was used as an outgroup to derive an evolutionary tree from this alignment (Fig. 6). It shows that inhibitors from the
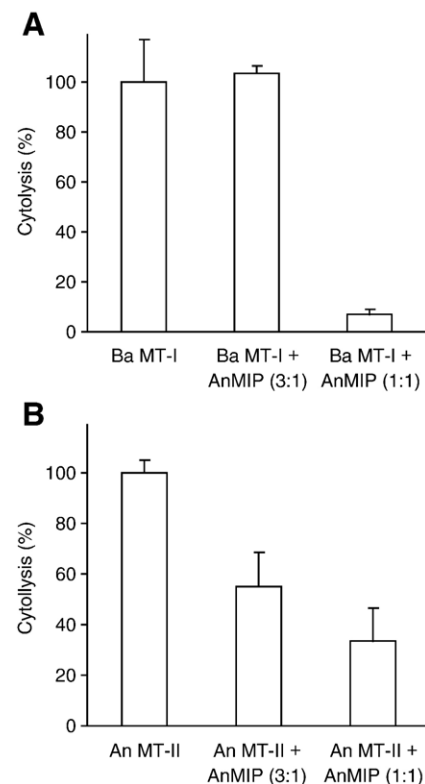
Fig. 3. Inhibition of the cytolytic activity of B. asper myotoxin I (A) and A. nummifer myotoxin II (B) on confluently growing myotubes, differentiated from the murine myoblast cell line C2C12 (ATCC CRL 1772), by AnMIP. Values are expressed relative to 0% cytolysis control (PBS) and 100% cytolysis control (0.1% Triton TX 100), as described in Materials and methods. The myotoxin:AnMIP molar ratios are indicated in parentheses. Bars represent mean±SD of duplicate cultures.

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accgcagagtttaaagatgcgtctgattctgctttccggtcttcttcttttgggaacctttctggccaacgga
          M  R  L  I  L  L  S  G  L  L  L  L  L  G  T  F  L  A  N  G

                                                      t
gatgagaaagattctgacgtacaaatgctgaattcgatgattgaagccgtaatgatacttcaaagagatttcgcc
 D  E  K  D  S  D  V  Q  M  L  N  S  M  I  E  A  V  M  I  L  Q  R  D  F  A   25

       t       c               t               g
aacctgagacatgccttgatgacagtccacacgcccgatcctttgggcgtggcagtgaaagattgtacgtgacc
 N  L  R  H  A  L  M  T  V  H  N  A  R  S  F  G  R  G  S  E  R  L  Y  V  T   50
          Y     F

aacaaggaagtcagtaagtttgaaggtctcgaagaaatttgtagccaagccgggggccatatcccttcccctcaa
 N  K  E  V  S  K  F  E  G  L  E  E  I  C  S  Q  A  G  G  H  I  P  S  P  Q   75

ctcgaaaatcagaacaaggccttcgaagatgttctggagaggcacaataaagcagcctaccttgtcgtgggtgac
 L  E  N  Q  N  K  A  F  E  D  V  L  E  R  H  N  K  A  A  Y  L  V  V  G  D  100

                                t
tcagcaaacttcaccaactgggctgcgggacaaccgaatgaggctgatggaacctgtgtgaaagcagatacacac
 S  A  N  F  T  N  W  A  A  G  Q  P  N  E  A  D  G  T  C  V  K  A  D  T  H  125
                            V

ggatcctggcactctgcgtcctgtgatgacaacctcttagtcgtgtgtgagttttatttcattttatgagaagga
 G  S  W  H  S  A  S  C  D  D  N  L  L  V  V  C  E  F  Y  F  I  L          147

agaccggc
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Fig. 4. cDNA nucleotide sequence (lowercase) and deduced amino acid sequence (uppercase) of AnMIP, clones 1 and 2 (DQ657241). Sequences of the primers used for the amplification are double underlined, and the initiation and termination codons are in boldface. The signal peptide is underlined. The differences found in clones 3 and 4 (DQ657242) relative to clones 1 and 2 are shaded in gray for the nucleotide sequence, and in black for their deduced amino acid sequences. The numbering corresponds to the mature protein sequence.

American crotaline species are divergent from those of *Trimeresurus* snakes, which are distributed only in Asia, and closer to the PLI from *Agkistrodon*, a genus present both in the Asian and American continents. Furthermore, the tree also shows that AnMIP is phylogenetically closest to CgMIP-II, the *C. godmani* PLIα inhibitor.
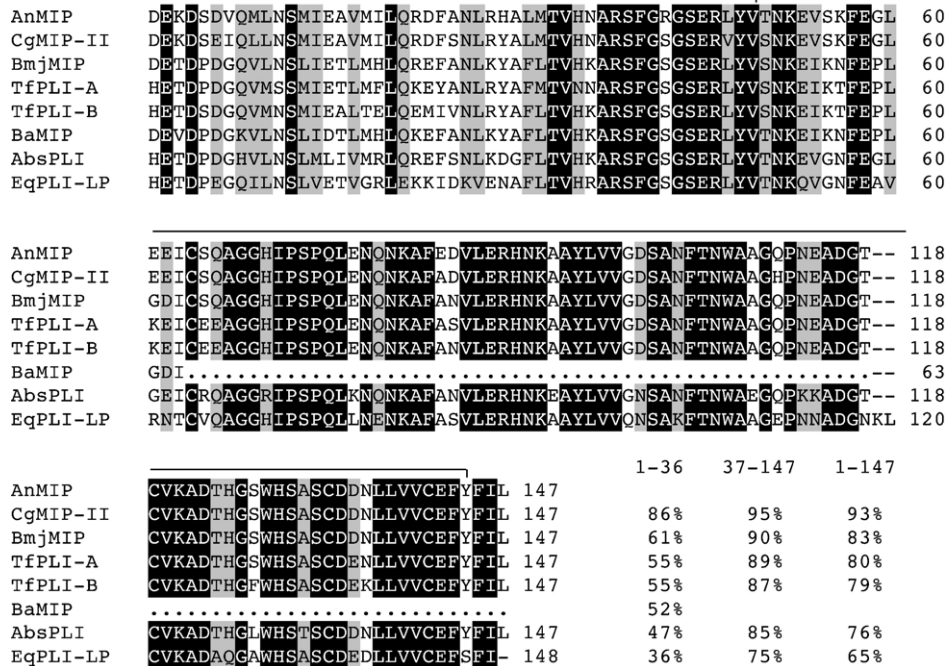
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AnMIP     DEKDSDVQMLNSMIEAVMILQRDFANLRHALMTVHNARSFGRGSERLYVTNKEVSKFEGL   60
CgMIP-II  DEKDSEIQLLNSMIEAVMILQRDFSNLRYALMTVHNARSFGSGSERVYVSNKEVSKFEGL   60
BmjMIP    DETDPDGQVLNSLIETLMHLQREFANLKYAFLTVHKARSFGSGSERLYVSNKEIKNFEPL   60
TfPLI-A   HETDPDGQVMSSMIETLMFLQKEYANLRYAFMTVNNARSFGSGSERLYVSNKEIKTFEPL   60
TfPLI-B   HETDSDGQVMNSMIEALTELQEMIVNLRYAFLTVHKARSFGSGSERLYVSNKEIKTFEPL   60
BaMIP     DEVDPDGKVLNSLIDTLMHLQKEFANLKYAFLTVHKARSFGSGSERLYVTNKEIKNFEPL   60
AbsPLI    HETDPDGHVLNSLMLIVMRLQREFSNLKDGFLTVHKARSFGSGSERLYVTNKEVGNFEGL   60
EqPLI-LP  HETDPEGQILNSLVETVGRLEKKIDKVENAFLTVHRARSFGSGSERLYVTNKQVGNFEAV   60

AnMIP     EEICSQAGGHIPSPQLENQNKAFEDVLERHNKAAYLVVGDSANFTNWAAGQPNEADGT--  118
CgMIP-II  EEICSQAGGHIPSPQLENQNKAFADVLERHNKAAYLVVGDSANFTNWAAGHPNEADGT--  118
BmjMIP    GDICSQAGGHIPSPQLENQNKAFANVLERHNKAAYLVVGDSANFTNWAAGQPNEADGT--  118
TfPLI-A   KEICEEAGGHIPSPQLENQNKAFASVLERHNKAAYLVVGDSANFTNWAAGQPNEADGT--  118
TfPLI-B   KEICEEAGGHIPSPQLENQNKAFANVLERHNKAAYLVVGDSANFTNWAAGQPNEADGT--  118
BaMIP     GDI...................................................--   63
AbsPLI    GEICRQAGGRIPSPQLKNQNKAFANVLERHNKEAYLVVGNSANFTNWAEGQPKKADGT--  118
EqPLI-LP  RNTCVQAGGHIPSPQLLNENKAFASVLERHNKAAYLVVQNSAKFTNWAAGEPNNADGNKL  120

                                            1-36   37-147   1-147
AnMIP     CVKADTHGSWHSASCDDNLLVVCEFYFIL  147
CgMIP-II  CVKADTHGSWHSASCDDNLLVVCEFYFIL  147    86%     95%     93%
BmjMIP    CVKADTHGSWHSASCDDNLLVVCEFYFIL  147    61%     90%     83%
TfPLI-A   CVKADTHGSWHSASCDENLLVVCEFYFIL  147    55%     89%     80%
TfPLI-B   CVKADTHGFWHSASCDEKLLVVCEFYFIL  147    55%     87%     79%
BaMIP     ...........................                52%
AbsPLI    CVKADTHGLWHSTSCDDNLLVVCEFYFIL  147    47%     85%     76%
EqPLI-LP  CVKADAQGAWHSASCDEDLLVVCEFSFI-  148    36%     75%     65%
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Fig. 5. Multiple sequence alignment for PLIαs using the CLUSTAL W algorithm. Identical residues are shaded in black, and conserved residues in gray. Conserved half cystines are in bold, and the CRD like domain is upper lined. Identity percentages relative to AnMIP (DQ657241) for the complete protein and the segments 1–36 and 37–147 are indicated for each inhibitor. The accession numbers and references for the proteins included in the alignment are described in Materials and methods.
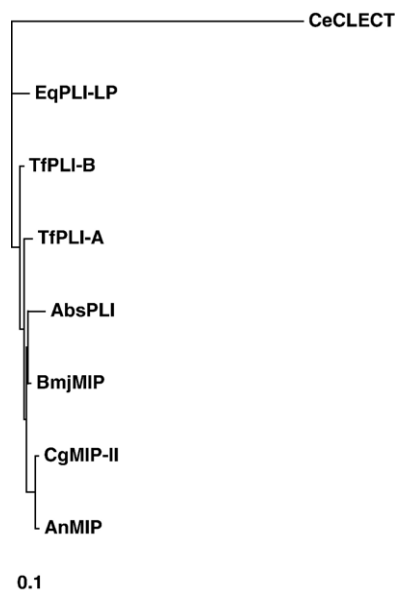
Fig. 6. Phylogenetic tree relating AnMIP to others PLIαs. The primary structures were aligned with CLUSTAL W, and the tree was built with PROTDIST and NEIGHBOR, using the sequence of *C. elegans* hypothetical protein *Y48E1B.9* (CeCLECT) as an outgroup. The accession numbers and references for the proteins included in the tree are described in Materials and methods.

Neither primers for the β or γ groups of PLIs achieved cDNA amplification, in attempts to search for such type of inhibitors in mRNA extracted from the liver tissue of *A. nummifer*.

4. Discussion

A new PLA$_2$ inhibitory protein was isolated from the plasma of *A. nummifer*, a crotaline snake from Central America. This inhibitor was named AnMIP, given its ability to neutralize the activity of myotoxic components of its own and related venoms. AnMIP presented physicochemical properties characteristic of PLIαs, as well as significant cross-reactivity with antibodies elicited against an α inhibitor (BaMIP). Further direct amino-terminal and cDNA sequencing confirmed its classification as a new member of the PLIα group.

The inhibitory profile of AnMIP is characteristic of PLIαs, with a specificity towards group II PLA$_2$s, either belonging to the catalytically-active (D49) or -inactive (K49) subtypes, exemplified in this study by *B. asper* myotoxin I and *A. nummifer* myotoxin II, respectively. In agreement with the inhibitory behavior of PLIαs, AnMIP did not affect the catalytic activity of taipoxin (group I), while it decreased only slightly that of bee venom PLA$_2$ (group III). This is in clear contrast to PLIγs, in particular those isolated from elapid snakes, which display a broad inhibitory profile against all three structural groups of venom PLA$_2$s (Lizano et al., 2003). Despite several attempts under controlled PCR conditions, no amplification of PLIβ or γ cDNAs from *A. nummifer* liver was achieved. However, this does not preclude the possibility of the presence of such types of inhibitors in this snake species.

Previous studies have reported PLIαs from American crotaline snakes to be composed of four to five subunits (Lizano

et al., 1997, 2000; Soares et al., 2003), the Asian crotaline protein TfPLIα to be a heterodimer (Inoue et al., 1991), and the Asian AbsPLIα and EqPLI-LP to be trimers (Ohkura et al., 1993; Okumura et al., 2003). Given this diversity of oligomeric structures, the subunit composition of the newly isolated AnMIP was studied. Results of analytical gel filtration in the native state, together with chemical cross-linking analyses, are consistent with a trimeric structure for AnMIP, in agreement with the model proposed by Okumura et al. (2005). This finding shows for the first time that a trimeric quaternary structure can be found in PLIαs of an American crotaline snake. The dissociation of subunits in SDS-PAGE under non-reducing conditions evidenced that the AnMIP trimer is held together by non-covalent forces, in similarity with the cases of AbsPLIα and EqPLI-LP (Ohkura et al., 1993; Okumura et al., 2003).

The full sequence of AnMIP was determined through cDNA cloning from liver mRNA. Three amino acid differences were found among the two sequences obtained by molecular cloning, at positions 29, 31, and 109. The first two differences are located in the most variable region of the mature protein, while the third lies in the conserved CTLD region. In all cases, however, the variations were conservative. Analytical isoelectrofocusing of the purified AnMIP protein resolved two bands, corresponding to estimated pI values of 4.1 and 4.7, respectively. Since the deduced amino acid differences found in the two cDNAs clones are conservative, this observation opens the possibility that glycosylation variants affecting the net charge of this protein may add to the observed microheterogeneity of AnMIP. Indeed, the sequence of AnMIP presented the known glycosylation site of PLIαs at position N103, and the protein was shown to be glycosylated, suggesting that the difference between the subunit molecular mass determined by ESI-MS and its predicted mass should be accounted by glycosylation.

Taken together, evidence from isoelectrofocusing, cDNA sequencing, and mass spectrometry, suggests the existence of AnMIP isoforms. It should be noted that the physicochemical characterization was performed on protein purified from pooled plasma of a few specimens, while cloning was performed from the liver of a single specimen. Both intraindividual and inter-individual microheterogeneity could explain the presence of isoforms. Similarly, previous reports have described isoforms in Abs PLIα(Okumura et al., 1999) and CgMIP-II (Lizano et al., 2000), based upon the sequencing of two cDNA clones in the former case, or the differences between deduced and direct N-terminal sequences in the latter. The presence of isoforms both in plasma and at the level of liver transcripts was also documented in the case of PLIγs, and it has been suggested that such minor structural changes may play a subtle role in the recognition of different PLA$_2$ variants (Dunn and Broady, 2001). In the same way, it is reasonable to assume that amino acid variability of AnMIP and other PLIαs could widen the range of interaction with diverse venom toxins.

Comparison of the primary structures of AnMIP and other PLIαs (Fig. 5) showed amino acid identity values in the range of 65 to 93%, the non-inhibitory PLI homologue of *E. quad-rivirgata* presenting the lower percentage. When only the more conserved region 37–147 was analyzed, identities ranging from

75 to 95% were obtained, whereas analysis of the first 36 residues resulted in scores ranging from 36 to 86%. In this more variable region, CgMIP-II presented a considerable identity (86%) to AnMIP, as compared to the other PLIαs (61% or lower). Interestingly, *C. godmani* myotoxin II presents 90% sequence identity to *A. nummifer* myotoxin II, being the most closely-related myotoxin by phylogenetic analysis (Angulo et al., 2002). On the other hand, BaMIP, a crotaline PLIα, presented a lower identity when a partial sequence was compared to AnMIP (52% for residues 1–36). This would be in agreement with the limited antigenic cross reactivity observed among these two proteins by enzyme immunoassay (46%).

It has been reported that PLIαs isolated from American crotaline snakes are more selective towards basic PLA$_2$s (Lizano et al., 1997, 2000), while those of Asian species preferentially interact with acidic PLA$_2$s (Inoue et al., 1997). Upon inspection of the aligned sequences of PLIαs, no evident amino acid changes could be ascribed to this reported difference in inhibitory profiles. An intraspecific specialization of the inhibitor towards particular PLA$_2$s co evolving in its own venom would seem a more reasonable explanation than an early subdivision of the specificity of these inhibitors in Asian and American crotalines. Nevertheless, this hypothesis remains to be addressed in future studies, as a larger number of inhibitors become available for the detailed characterization of their structure–function and evolutionary relationships.

Among the residues found only in AnMIP (V7, M9, R42, E84 for all clones, H29 for clones 1 and 2, and an V109 for clones 3 and 4), as compared to other PLIαs, four are located in the more variable region, being in most cases conservative of the amino acid properties. However, R42 and E84 introduce a positive and a negative charge, respectively, at positions were only serine and alanine, respectively, have been observed in other PLIαs.

In conclusion, a new myotoxin inhibitory protein, AnMIP, has been isolated from the plasma of the snake *A. nummifer* and classified as a PLIα, showing specificity towards group II PLA$_2$s from the venom of this and related crotaline species. Its complete primary structure was deduced by molecular cloning. Future studies will attempt its production in recombinant form to overcome the limited availability from the natural source, and to gain insight into the molecular mechanism(s) involved in its myotoxin inhibitory activity.

Acknowledgements

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