

## *Bothrops moojeni* myotoxin-II, a Lys49-phospholipase A<sub>2</sub> homologue: An example of function versatility of snake venom proteins<sup>☆</sup>

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### Abstract

MjTX-II, a myotoxic phospholipase A<sub>2</sub> (PLA<sub>2</sub>) homologue from *Bothrops moojeni* venom, was functionally and structurally characterized. The MjTX-II characterization included: (i) functional characterization (antitumoral, antimicrobial and antiparasitic effects); (ii) effects of structural modifications by 4-bromophenacyl bromide (BPB), cyanogen bromide (CNBr), acetic anhydride and 2-nitrobenzenesulphonyl fluoride (NBSF); (iii) enzymatic characterization: inhibition by low molecular weight heparin and EDTA; and (iv) molecular characterization: cDNA sequence and molecular structure prediction. The results demonstrated that MjTX-II displayed antimicrobial activity by growth inhibition against *Escherichia coli* and *Candida albicans*, antitumoral activity against Erlich ascitic tumor (EAT), human breast adenocarcinoma (SK-BR-3) and human T leukemia cells (JURKAT) and antiparasitic effects against *Schistosoma mansoni* and *Leishmania* spp., which makes MjTX-II a promising molecular model for future therapeutic applications, as well as other multifunctional homologous Lys49-PLA<sub>2</sub>s or even derived peptides. This work provides useful insights into the structural determinants of the action of Lys49-PLA<sub>2</sub> homologues and, together with additional strategies, supports the concept of the presence of others “bioactive sites” distinct from the catalytic site in snake venom myotoxic PLA<sub>2</sub>s.

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**Keywords:** *Bothrops*; cDNA cloning; Chemical modification; Myotoxin; Microbial; Parasitocidal and antitumoral activity; Phospholipase A<sub>2</sub>; Snake venom

**Abbreviations:** Basp, *B. asper* myotoxins; BnSP, *B. neuwiedi* myotoxins from São Paulo State; bp, base pair; BPB, 4-bromophenacyl bromide; BthTX, *B. jararacussu* bothropstoxins; CFU, colony-forming units; CK, creatine kinase; CNBr, cyanogen bromide; EDTA, ethylenediaminetetraacetic acid; MjTX, *B. moojeni* myotoxins; MjTX-II, myotoxin-II from *Bothrops moojeni* venom; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBSF, 2-nitrobenzenesulphonyl fluoride; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PrTX, *B. pirajai* piratoxin; RT-PCR, reverse transcriptase–polymerase chain reaction.

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## 1. Introduction

Phospholipases A<sub>2</sub> (PLA<sub>2</sub>s; EC 3.1.1.4) are widely distributed enzymes (Nevalainen et al., 2004a,b) of high medical-scientific interest due to their involvement in several inflammatory human diseases and envenomation by snake and bee venoms. PLA<sub>2</sub>s constitute a superfamily of different enzymes belonging to four groups on the basis of their source, amino acid sequence and biochemical characteristics (Six and Dennis, 2000; Murakami and Kudo, 2002). PLA<sub>2</sub>s from classes I, II and III have low  $M_r$  (13,000–18,000), are Ca<sup>2+</sup>-dependent and structurally different from class IV PLA<sub>2</sub>s. Those from class IV are intracellular, cytosolic, Ca<sup>2+</sup>-dependent and have high  $M_r$  (85,000). Recently discovered enzymes, either secreted (sPLA<sub>2</sub>s) or cytosolic (cPLA<sub>2</sub>s), were identified and did not clearly fit in any of the above classes. New classes were then proposed (Six and Dennis, 2000; Murakami and Kudo, 2002, 2004).

PLA<sub>2</sub>s from snake venoms have been classified as groups I and II on the basis of their primary structure and disulfide bridge pattern (Balsinde et al., 1999; Six and Dennis, 2000). In addition to their primary catalytic role, snake venom PLA<sub>2</sub>s show other important toxic/pharmacological effects including myonecrotic, neurotoxic, cardiotoxic, hemolytic, hemorrhagic, hypotensive, anticoagulant, platelet aggregation inhibition and edema-inducing activities (Gutiérrez and Lomonte, 1997; Ownby, 1998; Ownby et al., 1999; Soares et al., 2004a).

Muscular necrosis is a serious consequence of *Bothrops* snakebites that may lead to permanent loss of tissue or function and to require amputation of the affected member. Myonecrosis may be due to an indirect action as consequence of vessel degeneration and ischemia caused by hemorrhagic metalloproteases or by a direct effect of myotoxic PLA<sub>2</sub>s homologues on plasma membranes of muscle cells (Gutiérrez and Lomonte, 1997; Ownby, 1998; Ownby et al., 1999; Gutiérrez, 2002; Soares et al., 2004a).

During the last 15–20 years, there has been an increasing interest in the study of the myonecrotic venom components, resulting in the isolation and structural/functional characterization of several myotoxic PLA<sub>2</sub> homologues from *Bothrops* venoms (Soares et al., 2004a). Myotoxins isolated from these venoms belong to group IIA of PLA<sub>2</sub>s and may be subdivided into two subgroups: (i) Asp49 myotoxins with relatively low to moderate enzymatic activity and (ii) Lys49 myotoxins with practically no hydrolytic activity on artificial substrates (Gutiérrez and Lomonte, 1997; Ownby, 1998; Ownby et al., 1999; Soares et al., 2004a).

Myotoxin-I and -II homologues purified from *Bothrops moojeni* (MjTX-I and -II) are Lys49-PLA<sub>2</sub> and have been isolated and characterized (Lomonte et al., 1990; Soares et al., 1998, 2000a,b, 2004a). The structures of native MjTX-I and -II and of the complex MjTX-II-stearic acid have been recently solved (de Azevedo et al., 1997; Marchi-Salvador et al., 2005a; Watanabe et al., 2005). In order to study the function versatility, MjTX-II was functionally and structurally characterized. This characterization included: (i) parasiticidal, antimicrobial and antitumor effects; (ii) effects of chemical modifications by 4-

bromophenacyl bromide (BPB), cyanogen bromide (CNBr), acetic anhydride and 2-nitrobenzenesulphonyl fluoride (NBSF); (iii) inhibition by low molecular weight heparin, EDTA and PLA<sub>2</sub>-antibodies; and (iv) cDNA sequence and molecular structure of this myotoxin.

## 2. Materials and methods

### 2.1. Purification procedure

*B. moojeni* venom was purchased from Bioagents Serpentarium (Batatais-SP) and fractionated on CM-Sepharose column at pH 8.0 as previously described (Soares et al., 1998). Homogeneity was demonstrated by 12% SDS-PAGE, basic PAGE at 10%, isoelectric focusing, N-terminal amino acid sequencing, RP-HPLC and mass spectrometry (Soares et al., 2000a,b, 2003). Molecular weight of the MjTX-II was also determined by MALDI mass spectrometry. Far UV circular dichroism spectra (190–250 nm) were measured with a JASCO 810 (JASCO Inc., Tokyo, Japan) using 1 mm path length cuvettes and protein concentrations of 250 mg/mL for native or modified myotoxic PLA<sub>2</sub>s. In all cases, a total of three spectra were collected, averaged and corrected by subtraction of a buffer blank.

### 2.2. Chemical modification

Modification of histidine residues with 4-bromophenacyl bromide (BPB) was carried out as previously described (Soares et al., 2000a,b, 2001a,b) using 0.1 M ammonium bicarbonate buffer. Three milligrams of toxin were dissolved in 1 mL of 0.1 M ammonium bicarbonate containing 0.7 mM EDTA (pH 8.0) and 150 mL of BPB (0.8 mg/mL), and the mixture incubated for 24 h at 25 °C.

Modification of Lys residues with acetic anhydride (AA) was performed at a protein/reagent molar ratio of 1:50 (Soares et al., 2000a,b, 2001a,b). MjTX-II (3 mg) was dissolved in 1.5 mL of 0.2 M ammonium bicarbonate buffer at pH 8.0 plus 10 mL of AA and the mixture was incubated for 1 h at 25 °C.

Tyrosine residues were modified by treatment with 2-nitrobenzenesulphonyl fluoride (NBSF) as previously described (Soares et al., 2000a,b, 2001a,b). For that, 1 mmol of MjTX-II (9 mmol of Tyr) was dissolved in 14 mL of 0.1 M Tris-HCl (pH 8.0) and incubated with 9 mmol of NBSF for 20 h at 25 °C.

Modification of Trp residues was performed according to Soares et al. (2000a,b, 2001a,b). 9 mg of MjTX-II was dissolved in 4 mL 50% acetic acid containing 1 mg of *o*-nitrophenylsulphenyl chloride (NPSC) and incubated for 1 h at 25 °C. In all cases, excess reagent was removed by ultrafiltration through an Amicon YM-3 membrane and washed with water or 0.05 M ammonium bicarbonate (pH 8.0), followed by lyophilization.

### 2.3. Treatment of myotoxin-II with cyanogen bromide

A sample of 15 mg of MjTX-II was dissolved in 0.45 mL of 70% (w/w) formic acid and a 50-fold molar cyanogen bromide

over the methionine residues was added (Soares et al., 2000a,b, 2001a,b). The cleavage reaction developed under nitrogen for 24 h at room temperature. Control and cleaved myotoxin were applied on a Sephadex G-25 column (100×2.0 cm), which was equilibrated and eluted with 0.05 M, pH 8.0 ammonium bicarbonate.

#### 2.4. Myotoxic activity

Groups of five male Swiss mice (18–22 g) were injected in the right gastrocnemius muscle with MjTX-II/50  $\mu$ L PBS. After 1, 3 and 6 h, blood was collected from tail vessels in heparinized tubes and centrifuged for separation of the plasma. The amount of creatine kinase (CK) was then determined using 4  $\mu$ L plasma incubated for 3 min at 37 °C with 1.0 mL of the reagent according to the kinetic CK-UV protocol from Sigma Chem. Co. Activity was expressed in U/L, 1 unit corresponding to the production of 1  $\mu$ mol NADH/min (Soares et al., 2000a,b, 2001a,b).

#### 2.5. Edema-inducing activity

Groups of five male Swiss mice (18–22 g) were injected in the subplantar region with different doses of MjTX-II/50  $\mu$ L PBS. After 0.5 h, the paw edema was measured with a low pressure spring caliper (Mitutoyo-Japan) (Soares et al., 2000a,b, 2001a,b). Zero time values were then subtracted and the differences expressed in median %±S.D.

#### 2.6. Disruption of liposomes

Negatively charged liposomes (phosphatidylcholine, 63  $\mu$ mol; dicetylphosphate, 18  $\mu$ mol; and cholesterol, 9  $\mu$ mol) were obtained from Sigma Chem. Co. (Missouri, USA). The assay followed the procedure of Soares et al. (2000a,b, 2001a,b) on microplates, incubating 20  $\mu$ L of the liposome suspension and 20  $\mu$ L of MjTX-II solutions (in PBS) for 30 min at 37 °C or 4 °C.

#### 2.7. Cancer cell lines culture

Human breast (SK-BR-3) and acute T cell leukemia (Jurkat) cancer cell lines were maintained on RPMI 1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 10% fetal bovine serum, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. All cell culture reagents were purchased from Gibco. All cell lines were maintained at 37 °C in 5% CO<sub>2</sub> and 95% air with more than 95% humidity.

#### 2.8. Tumor cytotoxic activity

Tumor cytotoxic activity of PLA<sub>2</sub>s was assayed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining as described by Mosmann (1983). Tumor cells cultivated in appropriate flasks and maintained in continuously exponential growth were detached with 0.05% trypsin, 0.02%

EDTA in calcium-free phosphate-buffered saline (PBS) and washed three times with RPMI medium at 500×g/15 min/10 °C. Cells were disposed in 96-well plates at a density of 1×10<sup>5</sup> cells/well. After 24 h, the medium was removed and fresh medium, with or without different concentrations of indicated compounds (PLA<sub>2</sub> or methotrexate, 1–0.01 mg/mL), was added to the wells and incubated for 24 h (Roberto et al., 2004). Cytotoxic rate was calculated as follows: % of cytotoxicity of compounds = 1 – Abs drug treated / Abs control × 100.

In some experiments, cytotoxic activity was determined on Erlich ascitic tumor (EAT) cells grown in the peritoneal cavity of Swiss mice (Chwetzoff et al., 1989a). EAT cells were suspended in Tyrode-Ringer buffer (4×10<sup>6</sup> cells in the final volume of 1 mL) and incubated with several concentrations of PLA<sub>2</sub> (2–0.01 mg/mL) for 60 min. One hundred microlitres of Trypan Blue solution (1% in saline) were then added and the dead stained cells, as well as unstained cells, were independently counted using a haemocytometer.

#### 2.9. Microbicidal activity

*Escherichia coli* (ATCC 29648) and *Candida albicans* (ATCC 24433) were dispersed in 0.01 M sodium phosphate pH 7.4 buffer containing 1% peptone. These bacteria, harvested from fresh agar plates and adjusted to 4×10<sup>5</sup> colony-forming units (CFU)/mL, were utilized as a target for determining bactericidal activity. For that, 4×10<sup>5</sup> cells were incubated with varying amounts of MjTX-II, for 30 min at 37 °C, in PBS plus 1% peptone. Surviving bacteria were counted by the dilution plate technique as previously described (Soares et al., 2000a,b, 2001a,b).

#### 2.10. Leishmania strains and culture conditions

*Leishmania amazonensis* (MPRO/BR/72/M1841-LV-79), *L. braziliensis* (MHOM/BR/75/M2904), *L. major* (LV-39, clone 5-Rho-SU/59/P) and *L. donovani* (clone LV9-3 from MHOM/ET/67/HU3) strains were assayed on this study. Promastigote forms of all *Leishmania* species were grown in M199 medium (Gibco) supplemented with 40 mM Hepes (pH 7.4), 0.1 mM adenine, 7.7 mM haemin, 10% (v/v) heat-inactivated fetal calf serum, 50 U/mL penicillin and 50  $\mu$ g/mL streptomycin. The culture of parasites was incubated at 26 °C, keeping them at densities ranging between 5×10<sup>5</sup> and 3×10<sup>7</sup> parasites/mL (Kapler et al., 1990). Viability was evaluated from motility and cell density was determined using a haemocytometer.

#### 2.11. Cytotoxic effect of the MjTX-II on Leishmania viability

The direct cytotoxic effect of purified MjTX-II against *Leishmania* species was measured. Parasites (3×10<sup>6</sup>/well) were incubated in M199 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) in the presence or absence of myotoxin (0.1–100  $\mu$ g/mL) for 4 h, then pulsed with 0.5  $\mu$ Ci/well [<sup>3</sup>H]thymidine, and the incorporation of radioactivity by viable parasites was determined after 16 h using a  $\beta$ -counter.

### 2.12. Effect of the MjTX-II on *Schistosoma mansoni*

Adult Balb/c mice were divided into 7 groups with 10 animals each. Six groups were inoculated subcutaneously with *S. mansoni* using 60 cercariae/mouse and the remaining group was maintained without infection. Among the six infected groups, four were treated with MjTX-II, as described: (i) two groups received doses of 100 µg on days 0 and 20 after inoculation of the cercariae and (ii) other two groups received 50 µg of MjTX-II also on days 0 and 20. At the end of the 8th and 10th weeks after infection, all mice were submitted to fecal examination using the Kato-Katz technique.

### 2.13. Other activities

Platelet aggregation was measured turbidimetrically using washed rabbit platelets and a Whole Blood Lumi-Aggregometer as previously described (Andrião-Escarso et al., 2002). Phospholipase activity was evaluated using egg yolk phospholipids as substrate (Andrião-Escarso et al., 2000, 2002). The recalcification time test of citrated sheep platelet poor plasma was used to determine the anticoagulant effect (Soares et al., 2001a,b). Lethality induced by native and modified MjTX-II was evaluated by i.p. injection (Soares et al., 2000a,b, 2001a,b). Neurotoxic activity was assayed as previously described (Rodrigues et al., 2004).

### 2.14. Activity inhibition

Inhibition by low molecular weight heparin (Fragmin®, 25,000 IE/mL,  $M_r$  5000) was evaluated after incubation for 30 min at 37 °C, molar ratio heparin/toxin=2:1. EDTA was used at 1 mM.

### 2.15. cDNA cloning, sequencing and computer sequence analysis

The N-terminal primer was constructed on basis of the N-terminal sequence of MjTX-II with some degenerated positions (5' TTAAGCTTAGCCTGYTNGARYTGGGG3') and of complementary C-terminal sequences of all Lys49 PLA<sub>2</sub>s homologues (5' TAGAATTCGA GGGTTTTTCCCGGCC3'—*EcoRI* restriction site underlined) (GIBCO-BRL). The GIBCO-BRL procedure was performed, using TRIZOL for the extraction of total RNA from *B. moojeni* venom glands. The first cDNA strand was synthesized starting from 5 µg total RNA in the presence of Um-MLV reverse transcriptase and oligo(dT) for 1 h at 37 °C. Forty cycles were performed, each one consisting of: one denaturation step, 1 min at 95 °C; one primer annealing step, 2 min at 42 °C; and one extension, 1.5 min at 72 °C. The RT-PCR product was identified, purified and cloned on Ready-To-Go TM pUC18 Smal/BAP (Pharmacia-Biotech). The size of the insert (cDNA) was evaluated on a 1% (w/w) agarose gel after digestion of pUC18 plasmid with *EcoRI* and *HindIII* enzymes. The dideoxy chain termination method in both strands was used with the aid of an automatic DNA sequencer (ALF Express, Pharmacia-Biotech). Nucleotide and

amino acid sequences were aligned with those taken from the Gene Bank (National Center for Biotechnology Information, USA) and Swiss-Prot. Identification and comparison of these sequences were carried out through BLAST (Basic Local Alignment Search Tool-NCBI). The cDNA sequence was recorded in the GenBank under no. AF145759 and SwissProt under no. Q9I834.

### 2.16. Crystal structure analysis of MjTX-II

The MjTX-II crystal structure was previously determined to a resolution of 1.8 Å (Watanabe et al., 2005). This structure was analysed such that each amino acid residue type was ranked according to the solvent exposed area of the side chains as calculated using WHATIF program. Chemical modification of highly exposed residues was assumed to be more probable than buried residues and this analysis allows possible target residues identification for the several modifications. These possible target sites were subsequently localized in the three-dimensional structure and displayed using RIBBONS program (Carson, 1997).

## 3. Results and discussion

Structure–function relationships of several snake venom myotoxins, which are PLA<sub>2</sub>s homologues have been extensively investigated (Kini, 1997, 2003). Aiming at the elucidation of the action mechanism of these enzymes, several attempts have been described such as chemical modifications of specific amino acids (Soares and Giglio, 2003), site directed mutagenesis (Chioato and Ward, 2003), X-ray crystallographic techniques (Murakami and Arni, 2003), NMR, spectrofluorimetry, complex formation with inhibitors and natural or artificial substrates (Kini, 1997; Ward et al., 1998a,b).

The Lys49 myotoxin inactivation upon artificial substrates, belonging to class II of Crotalinae snake PLA<sub>2</sub>s (Maraganore et al., 1984; Maraganore and Henrikson, 1986), suggested the presence of a “toxic” site distinct from the catalytic site of these proteins. Several Lys49 myotoxins have been structurally characterized (Arni and Ward, 1996; Ward et al., 1998b; Magro et al., 2003; Soares et al., 2004a; Watanabe et al., 2005) and attempts to define the toxic and pharmacological effects performing region(s) have been reported (Ownby et al., 1999; Soares and Giglio, 2003; Soares et al., 2004a).

MjTX-II induces muscle fibres myonecrosis, evidenced by light intravital microscopy, and dose-dependent CK liberation (Fig. 1A); this myotoxin is cytotoxic to muscle cells (Fig. 1B), induces blockage of the neuromuscular junction (Fig. 1C) and formation of time depending edema in mice (Fig. 1D). MjTX-II also disrupts negatively charged liposomes in a dose–temperature-dependent manner (Fig. 1E) and shows toxicity by i.p. route at ~8 mg/kg. MjTX-II does not display anticoagulant or PLA<sub>2</sub> activity upon egg yolk.

PLA<sub>2</sub>s are multifunctional proteins able to participate as mediators in several inflammatory diseases and can be used in some applied areas of medicine, as detection of severe pre-eclamps, general anesthetic action, treatment of rheumatoid



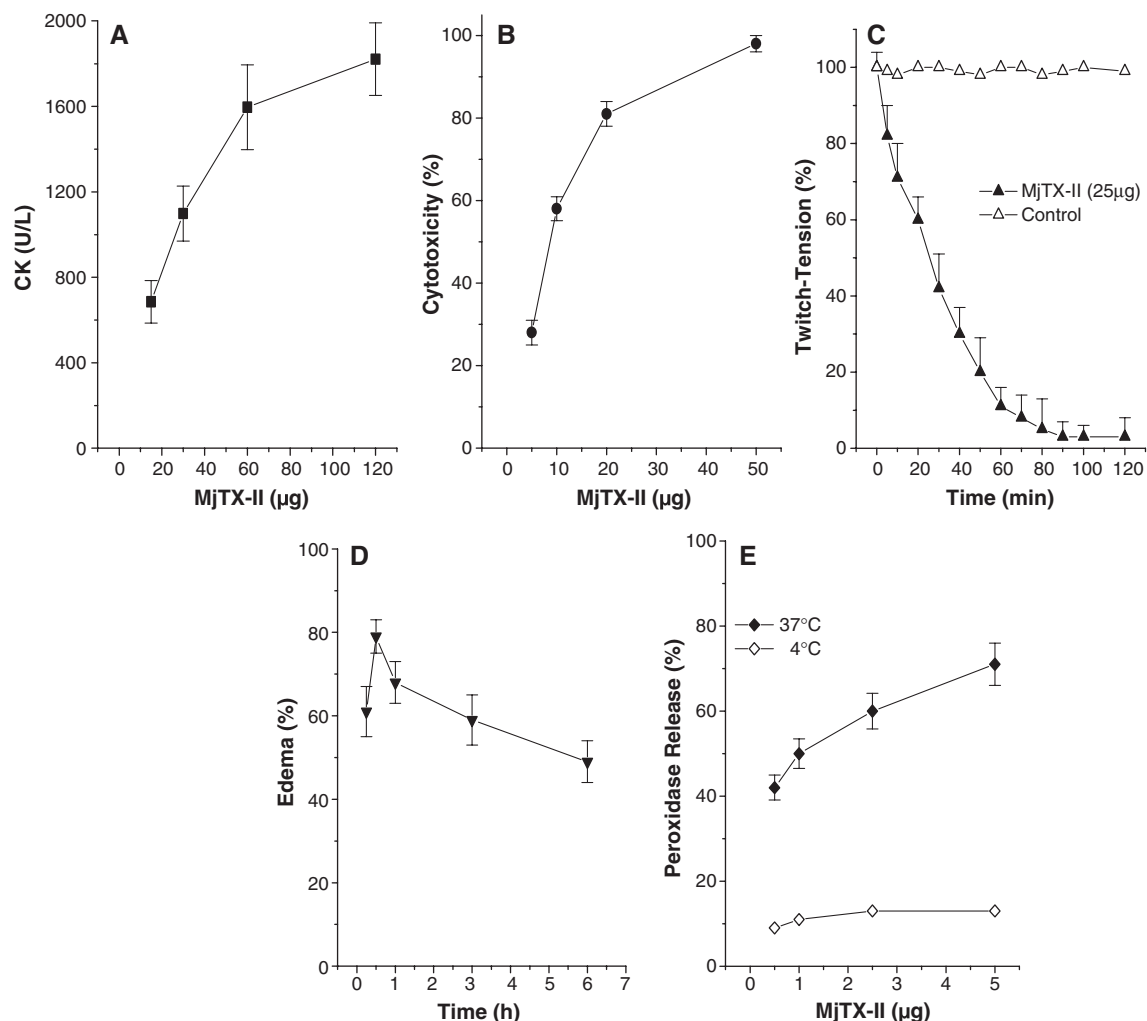


Fig. 1. Biological activities induced by *B. moojeni* MjTX-II. (A) Myotoxic activity of *B. moojeni* MjTX-II (20–120 µg) in mice. Plasma creatine kinase (CK) increases after the intramuscular injection of toxin. (B) The cytotoxic effect of MjTX-II (5–50 µg) on the C2C12 myoblast/myotubes cells was estimated by the release of lactate dehydrogenase (LDH) to the supernatant, 3 h after exposure of cells to samples. (C) Neurotoxicity activity of *B. moojeni* MjTX-II (25 µg) on nerve–muscle preparation of the mice. Results are presented as means±S.D. ( $n=4$ ). (D) Time-dependent edema induced by 50 µg of MjTX-II after the injection in the paw of 18–22 g male Swiss mice. (E) Peroxidase release from liposomes incubated at 4 °C or 37 °C for 30 min with MjTX-II. Release is expressed as percentage, with 100% as the peroxidase release from liposomes incubated with 0.2% Triton X-100. Phosphate-buffered saline (PBS) was included as a negative control in all experiments. Results are presented as means±S.D. ( $n=6$ ).

arthritis, as bactericidal agents in lachrymal glands and other tissues, as a new class of HIV inhibitors by blocking the host cell invasion and as potential antimalarial agents (Soares et al., 2004a).

MjTX-II also displayed antimicrobial activity inhibiting growing of *E. coli* and *C. albicans* cell lines when incubated for 30 min (Fig. 2A) and antitumoral activity against some human and mice cell lines (Fig. 2B). In addition to its lethal effect on fungi, bacteria and tumor cells, MjTX-II is effective as parasitocidal agent against *Leishmania* sp. (Fig. 2C) and *S. mansoni* (Table 1). Its anti-*S. mansoni* activity was characterized and further studies are in progress. Against *S. mansoni*, the toxin promotes dissociation of the couple, oviposition inhibitory effect and death of the parasite. A quantitative reduction of *S. mansoni* eggs/gram of feces was observed in mice treated with MjTX-II (50 or 100 µg) on the 0th and 20th day after infection (Table 1). This is another promising future therapeutic

application of these multifunctional proteins or even for a derived peptide.

The antitumor, parasitocidal and microbicidal activities of MjTX-II were independent on its catalytic activity, since this Lys49 myotoxin is catalytically inactive. Some snake venom PLA<sub>2</sub>s show antitumor activity over different cell lines, as *Crotalus durissus terrificus* crotoxin on murine erythroleukemia cells in vitro and for patients with solid tumors (Corin et al., 1993; Cura et al., 2002), *Bothrops asper* basic myotoxic PLA<sub>2</sub> (myotoxin-III) on mouse adrenal tumor cells (Bultrón et al., 1993) and *Naja naja naja* acidic PLA<sub>2</sub>s on Ehrlich ascites tumor cells (Rudrammaji and Gowda, 1998). We propose that the cytotoxic activity on tumor cell lineages observed in this work is associated with apoptosis induction. This hypothesis was based on the fact that PLA<sub>2</sub> enzymes have been proposed to play a role mediating apoptosis in various models, including tumor cell lines (Cummings et al., 2000). Paramo et al. (1998) showed that

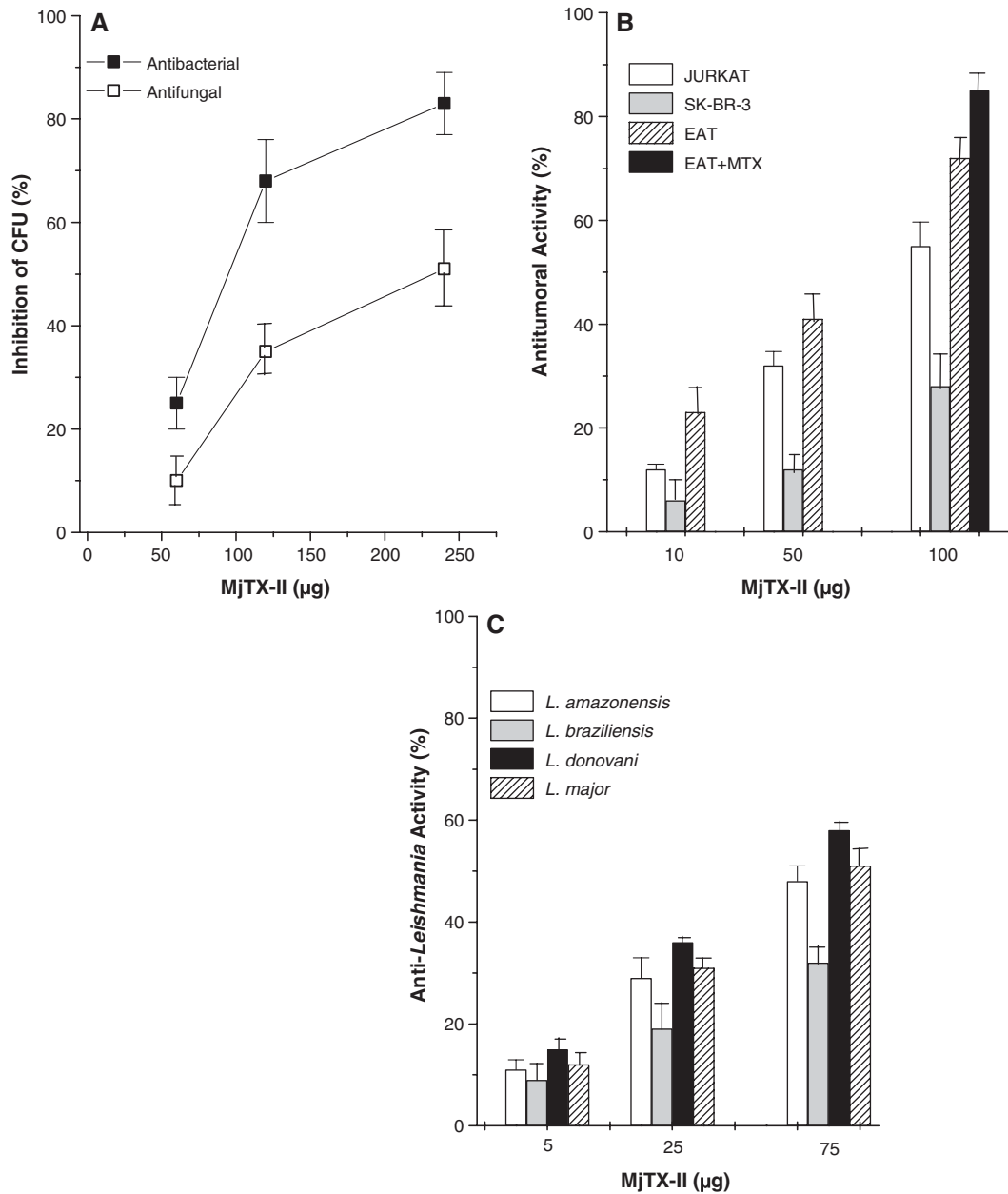


Fig. 2. Functional biological applications induced by *B. moojeni* MjTX-II. (A) Microbicide activity induced by different concentrations of MjTX-II upon *E. coli* (■) and *C. albicans* (□). (B) Antitumoral activity of MjTX-II upon different cell lines. Different concentrations (10, 50 and 100 μg) of myotoxin were incubated with human breast (SK-BR-3), acute T cell leukemia (JURKAT) and Erlich ascitic tumors (EAT) cells lines. Methotrexate (MTX, 100 μg) was used positive control. (C) Cellular viability of *L. amazonensis*, *L. braziliensis*, *L. donovani* and *L. major* after treatment with MjTX-II (5, 25 and 75 μg). Cytotoxicity was expressed as percentage (%). Results are presented as means±S.D. ( $n=6$ ).

*B. asper* myotoxin-II (Lys49) and -III (Asp49) were lethal for a variety of bacteria. Since Basp-II (Lys49) is catalytically inactive, the bactericidal effect is likely to be independent on this activity. Its 115–129 C-terminal peptide reproduced the bactericidal effect of the whole molecule.

The sequence of MjTX-II obtained from a 366pb cDNA fragment is able to codify for a mature 122 amino acid residues protein, confirmed by direct sequencing of the first N-terminal 53 residues (Fig. 3A). The complete sequence was used for the refinement of the crystal structure at 1.8 Å resolution (Watanabe et al., 2005).

The primary structure of MjTX-II shows high homology with other Lys49 snake venom myotoxins (Fig. 3B), with an insertion of bases which codify for an Asn, at position 120, not usually found in all other PLA<sub>2</sub>s. The amino acids involved with the catalytic site of Lys49 PLA<sub>2</sub>s are also conserved in MjTX-II, Asn28, Gly30, Val31, Leu32, Gly33, His48, Lys49 and Asp99. Inspection of the primary structures of the myotoxic Lys49 isoforms MjTX-I and MjTX-II revealed 20 substituted residues, including: Asn60↔Gly60, Asp76↔Ser76, Glu77↔Asn77, Lys110↔Leu110, Gly111↔Asp111, Arg117↔Tyr117, Asp126↔Lys126 and Asp131↔Pro131. These 20 mentioned

Table 1  
Results from the feces examinations between the 8th and 10th weeks after infection with *S. mansoni*, in the mice treated with MjTX-II and untreated mice

Weeks	Untreated	Treatment (days/dose)			
		0		20	
		50 µg	100 µg	50 µg	100 µg
8th	245	94	48	16	0
10th	215	81	23	8	0

changes might be involved with the differences reported for the neuromuscular and myotoxic effects on these toxins. MjTX-II induced post-synaptic blockage on mice synaptosome preparations, while MjTX-I did not show any effect. Both myotoxins showed the “inverted pre-synaptic” site (Ward et al., 1998a), Asn86/Lys93; however, no pre-synaptic effect was observed on neuromuscular preparations.

MjTX-II showed toxicity 10% to 15% more effectively than MjTX-I and this difference might be explained by the higher basicity of the C-terminal region of MjTX-II. Inspection of this region, supposed to be responsible for part of the myotoxic effect of *B. asper* Lys49 Basp-II, shows that MjTX-I displays a net charge +3 in this region at pH7.4 compared to +6 in MjTX-II and +6 or +7 in other Lys49 myotoxins (Soares et al., 2004a).

Chemical treatment of proteins may result in modification of multiple residues due either to the occurrence of a given amino acid at various positions in the protein or to non specific chemical reactions. Experiments were therefore performed to evaluate the number and specificity of residues that were modified by each chemical treatment.

Amino acid analysis comparisons of native and modified MjTX-II showed that single His and two Tyr residues were modified by BPB and NBSF, respectively. The dimeric MjTX-II crystal structure is shown as a ribbon diagram with the side chains of residues, which underwent chemical modifications (Tyr52, Tyr119, His48 and Trp77) in a ball-stick representation for a monomer (Fig. 4).

Basic PAGE electrophoresis demonstrated that acetylation of Lys residues strongly reduced the toxins basicity since they did not migrate in the gel after treatment with acetic anhydride. Only 50% of the Trp residues were modified by NPSC, since A365nm of the NPS-toxin was about 50% when compared with NPS-Trp. Identification of the N-terminal residue of the fractions from the Sephadex G-25 column after treatment of MjTX-II with CNBr revealed Ser-1/Asp-1 for the shorter chains and Ile-1/Ile-1 for the longer chains (Ile-9 in the protein), respectively. Circular dichroism spectra of the native and modified MjTX-II demonstrate defined minima at 211 and 222 nm, indicating a strong  $\alpha$ -helical contribution to the CD signal, which agrees with the high  $\alpha$ -helical content. The majority of the chemical modifications did not result in significant alterations of the spectra, which suggests that the secondary structure of the proteins remains largely unchanged. However, cyanogen bromide treatment resulted in a wavelength shift of the first minimum to 207 nm with a decreased minimum value at 222 nm, which indicates that

secondary structural modification is associated with cleavage of the N-terminal octapeptide.

Chemical modification of Met residues by carboxymethylation, as well as of His with BPB, Trp with NPSC, Tyr with NBSF and Lys by acetylation, affected differentially its pharmacological or toxic activities (Table 2). The neuromuscular activity was almost fully abolished by these modifications. Liposome disrupting activity was not significantly affected by these modifications. Cleavage of the N-terminal octapeptide from MjTX-II largely reduced its lethal, myotoxic, neurotoxic and edema-inducing activities, but only partially the liposome disrupting and cytotoxic effects.

Carboxymethylation of MjTX-II Met-8 reduced its myotoxic and lethal effects. Met-8 is highly conserved in snake venom PLA<sub>2</sub>s. Carboxymethylation of Met-8 generates a variant with 2% of the enzymatic activity and less than 3% of the lethal activity of native Pa-11 from *Pseudechis australis* venom (Takasaki et al., 1990); it had about 5% of the original ability to block directly and indirectly stimulated mouse synaptosome preparations.

The highly conserved His48 in PLA<sub>2</sub>s participates in catalysis (Scott et al., 1992). Alkylation of this residue induces loss of the hydrolytic activity on phospholipids and reduction of the toxic and pharmacological effects of PLA<sub>2</sub>s (Soares and Giglio, 2003).

The first report of a PLA<sub>2</sub> chemically modified with His48 was bovine pancreatic PLA<sub>2</sub> (Volwerk et al., 1974). This enzyme, when alkylated with *p*-bromophenachyl bromide (BPB), loses completely its enzymatic activity. Alkylation of MjTX-II His48 with BPB reduced its myotoxic, lethal and neurotoxic effects, and, in a smaller level, the edema-inducing and liposome disruption effects, thus suggesting a dissociation or partial overlapping of the pharmacological and toxic effects. The cytotoxic and bactericidal activities are not affected, thus corroborating the hypothesis that the functional site for these effects is the C-terminal domain and not the catalytic site. The exact mechanism by which BPB disturbs the toxic and pharmacological properties of the Lys49-PLA<sub>2</sub>s myotoxins remains to be determined. An acidic Asp49-PLA<sub>2</sub> from *Bothrops jararacussu* was crystallized (Takeda et al., 2004) and the structure was solved (Magro et al., 2005) showing tertiary and quaternary structural changes. The Lys49-PLA<sub>2</sub> BthTX-I chemically modified by BPB was recently crystallized by us (Marchi-Salvador et al., in preparation).

Treatment of *B. moojeni* and other similar venoms with BPB is a strategy to attenuate their toxic effects, making possible their better utilization for the production of anti-*Bothrops* or anti-PLA<sub>2</sub> serum, since higher doses of antigens could be administered without causing extensive damage to the inoculated animals (Soares et al., 2004b).

The recurrence of nine invariant Tyr residues in almost all venom phospholipases A<sub>2</sub> suggests that they are responsible for some important function in the molecule (Soares and Giglio, 2003; Soares et al., 2004a). *p*-Nitrobenzenesulfonyl fluoride (NBSF) was found to be a specific, mild reagent for the modification of tyrosine residues in proteins. Tyr residues are partially responsible for the myotoxic and neurotoxic effects of

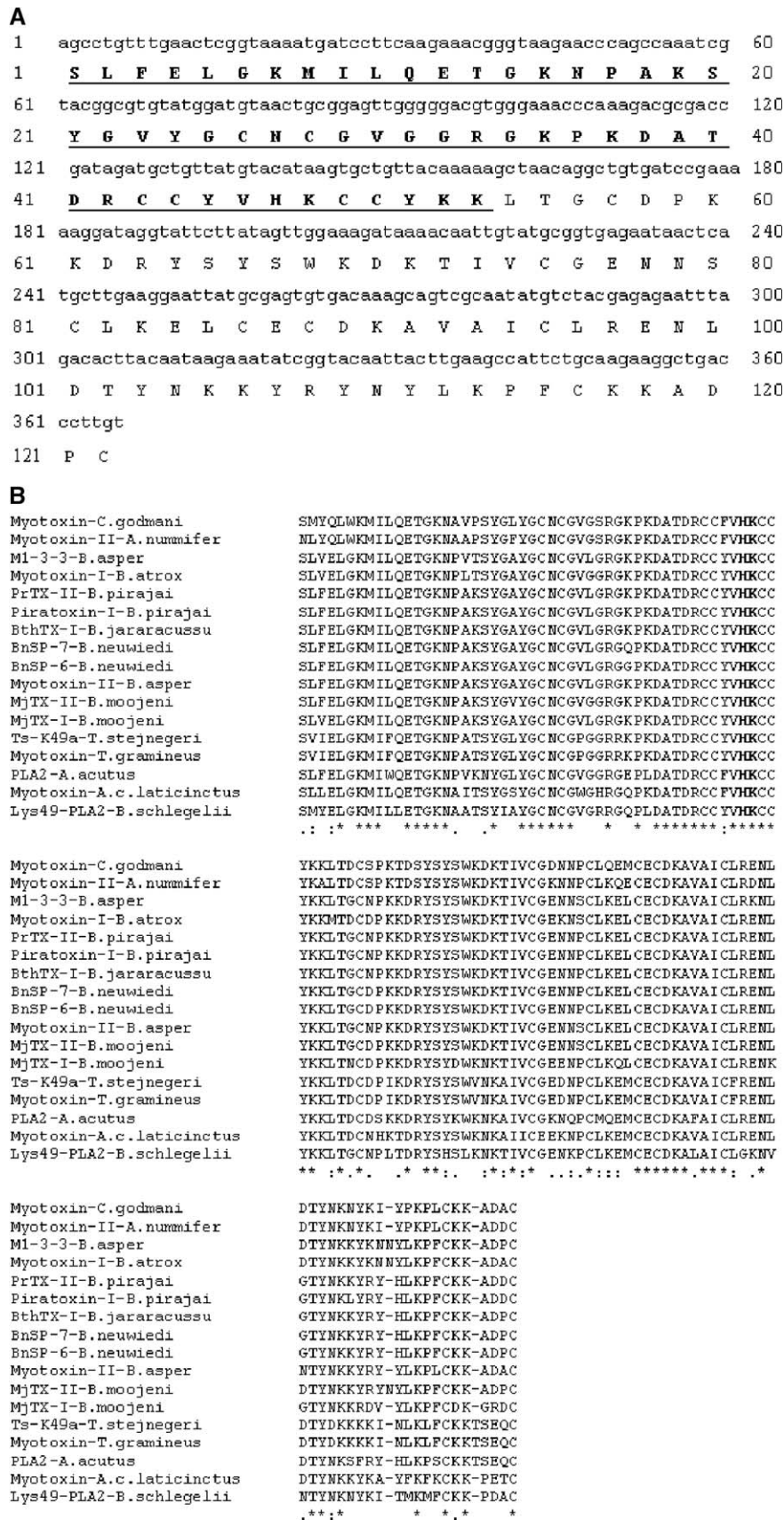


Fig. 3. Primary sequence of *B. moojeni* MjTX-II. (A) The cDNA and deduced amino acid sequences of *B. moojeni* MjTX-II. The sequence of the toxin was confirmed by the direct sequencing of the first 52 amino acid residues underlined (Soares et al., 1998). (B) Alignment of amino acid sequence of *B. moojeni* MjTX-II and other PLA<sub>2</sub>s-like toxins. Gaps were introduced to maximize the sequence homology. Alignments were made according to the half-cystine residues (bovine pancreatic PLA<sub>2</sub> has an 11–77 instead of the 50–133 disulfide bridge).



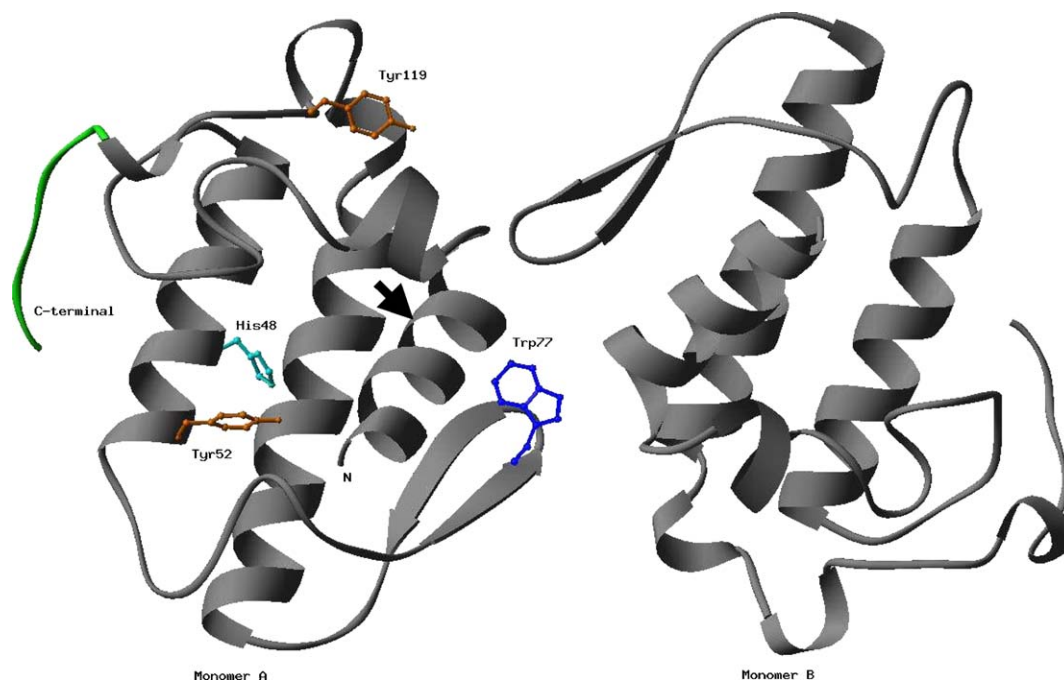


Fig. 4. Crystal structure of *B. moojeni* MjTX-II. The dimeric MjTX-II crystal structure (Watanabe et al., 2005) is shown as a ribbon diagram with the side chains of residues which underwent chemical modifications (Tyr52, Tyr119, His48 and Trp77) in a ball-stick representation (Carson, 1997). The C-terminal region is shown in green and the position of the N-terminal octapeptide cleavage with CNBr indicated by arrow.

MjTX-II and, in a lesser extension, for other effects. The two Tyr residues (Tyr52 and Tyr119) with the highest exposed surface areas in the MjTX-II crystal structure are probably those which were chemically modified and therefore participant of the myotoxic and neurotoxic activities of this toxin.

Modification of Trp reflected only in the neuromuscular effect caused by MjTX-II, and therefore this residue is likely to have a relevant participation in this activity and/or this chemical modification can interfere in the stability of the interaction between the monomers of this dimeric toxin. Trp77 is known to be useful to maintain this homodimeric interaction. Acetylation of Lys residues demonstrated that basic character is important for the toxic effects (myotoxic, cytotoxic and lethal activities). Recently, Angulo et al. (2005) demonstrated that the cytolytic activity of various Lys49-PLA<sub>2</sub> homologues in vitro, as well as their myotoxic activity in vivo are reduced, although not abolished, at pH 5.0, when compared with pH 7.2. This suggests that the switch from the dimeric to the monomeric forms of

these myotoxins may lead to a reduced ability to disrupt plasma membranes.

Cleavage of the N-terminal octapeptide of MjTX-II seems to be very relevant for all pharmacological effects, corroborating the hypothesis that interaction between the N-terminal region and the membrane is the first step of recognition, which precedes PLA<sub>2</sub>'s action. Some snake venom PLA<sub>2</sub>s have been treated with cyanogen bromide to cleave the NH<sub>2</sub>-terminal region (Chwetozoff et al., 1989a,b), since Met-8 is highly conserved in many of these enzymes (Ward et al., 1998a,b; Ownby et al., 1999). This region is also antigenically important in some snake PLA<sub>2</sub>s, since removal of the N-terminal octapeptide affects its reactivity against monoclonal and polyclonal antibodies (Díaz et al., 1994; Angulo et al., 2001).

Due to this functional diversity, these structurally similar proteins arose the interest of many researchers as molecular models for studies of structure–function relationships. One of the employed strategies for the relationship between catalytic activity and toxic/pharmacological effects of PLA<sub>2</sub>s elucidation is based on the evaluation of these parameters variations, following chemical modifications of specific amino acid residues (His, Met, Lys, Tyr, Trp and others) of the enzyme. Using this approach, a dissociation of pharmacological effects and enzymatic activity has been observed. Overall, specific amino acid modification studies, although failing to pinpoint the region of the PLA<sub>2</sub> molecule responsible for pharmacological activities, complies with the separate sites determining catalytic and pharmacological activities hypothesis (Soares and Giglio, 2003).

Bactericidal, neurotoxic and cytotoxic effects of MjTX-II are fully abolished with heparin incubation. It is known that heparin binds to the C-terminal (115–129) of *B. asper* myotoxin-II, and

Table 2  
Biological effects of *B. moojeni* native (N) or modified (M) MjTX-II

Myotoxin	LD <sub>50</sub> (mg/kg)	Myotoxicity (%)	Edema (%)	Cytotoxicity (%)	Release peroxidase (%)
MjTX-II	7.6±0.83	100	100	96	95.6
Met	45.8±0.73	22.9	47.2	90	78.3
His	45.2±0.38	44.6	87.9	53	91.2
Trp	12.2±0.71	84.5	86.1	100	94.3
Tyr (s)	22.5±0.48	60.7	91.3	72	92.6
Lys (s)	45.8±0.68	23.8	22.4	5.0	32.8
CNBr	45.6±0.51	25.7	24.2	82	49.8
Hep+MjTX-II	10.1±0.59	48.6	77.4	1.0	nd

nd, not determined.

that this region is related to its cytotoxic, myotoxic and bactericidal effects, with a probable contribution of Lys36 and Lys38 residues (Lomonte et al., 2003; Soares et al., 2004a). Incubation of MjTX-II with heparin completely inhibited the cytotoxic and bactericidal activities (Table 2), but only partially the myotoxic, edema-inducing and lethal effects. Incubation with EDTA did not significantly reduce any of the above effects (results not shown). Since homology between MjTX-II and Basp-II is near ~98% in these regions, the C-terminal region obviously also plays a key role in the membrane damaging effect of MjTX-II, including neurotoxic, cytotoxic and bactericidal activities.

Snake venom PLA<sub>2</sub>s are multifunctional proteins with promising biotechnological applications. Isolation and functional characterization of these enzymes will provide better insights of their mechanism of action as well as an interesting tool to study structure–activity relationships and open the possibility to use these proteins as molecular models for treatment of diseases in the future.

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