



Identification of linear B-cell epitopes on myotoxin II, a Lys49 phospholipase A₂ homologue from *Bothrops asper* snake venom

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ABSTRACT

Knowledge on toxin immunogenicity at the molecular level can provide valuable information for the improvement of antivenoms, as well as for understanding toxin structure–function relationships. The aims of this study are two-fold: first, to identify the linear B-cell epitopes of myotoxin II from *Bothrops asper* snake venom, a Lys49 phospholipase A₂ homologue; and second, to use antibodies specifically directed against an epitope having functional relevance in its toxicity, to probe the dimeric assembly mode of this protein in solution. Linear B-cell epitopes were identified using a library of overlapping synthetic peptides spanning its complete sequence. Epitopes recognized by a rabbit antiserum to purified myotoxin II, and by three batches of a polyvalent (Crotalidae) therapeutic antivenom (prepared in horses immunized with a mixture of *B. asper*, *Crotalus simus*, and *Lachesis stenophrys* venoms) were mapped using an enzyme-immunoassay based on the capture of biotinylated peptides by immobilized streptavidin. Some of the epitopes identified were shared between the two species, whereas others were unique. Differences in epitope recognition were observed not only between the two species, but also within the three batches of equine antivenom. Epitope V, located at the C-terminal region of this protein, is known to be relevant for toxicity and neutralization. Affinity-purified rabbit antibodies specific for this site were able to immunoprecipitate myotoxin II, suggesting that the two copies of epitope V are simultaneously available to antibody binding, which would be compatible with the mode of dimerization known as “conventional” dimer.

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1. Introduction

Serotherapy, discovered over a century ago, stands amongst the significant medical contributions of immunology, saving thousands of patients who suffer from envenomings every year (Bon, 1996; Kasturiratne et al., 2008; Harrison et al., 2009). Therapeutic antivenoms are conventionally prepared by injecting one or several venoms as immunogens in animals, aiming to elicit high levels of antibodies that bind to and neutralize their most relevant toxins, if not all. Therefore, elucidating the immunological

properties of toxic venom components is a medically relevant task (Ménez, 1985).

Immunological characteristics of venoms can be analyzed at various levels, i.e. focusing on either whole venom secretions, individual toxin molecules, or their submolecular antigenic sites. Considering whole toxin molecules as “study units”, features such as their immunogenicity, antigenic cross-reactivity, and neutralization by antibodies contained in specific or paraspecific antisera can be evaluated. Further, immunological properties of toxins can be analyzed at a molecular level, by studying details of their fine antigenic structure (Ménez, 1985). Such studies aim to delineate toxin epitopes for B and T lymphocytes of immunized animals, to identify which

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epitopes are recognized by neutralizing antibodies, and to elucidate the molecular mechanisms that underlie such neutralization. All these types of approaches, i.e. focusing on crude venoms, on toxin molecules, or on their submolecular antigenic structures, provide useful information for the rational design, development, and clinical use of antivenoms (Ménez, 1985; Dias da Silva et al., 1989; Wagstaff et al., 2006; Stock et al., 2007; Espino-Solis et al., 2009; Gutiérrez et al., 2009; Calvete et al., 2009; Calvete, 2010).

Although the antigenic structure of some snake venom toxins, for example the α -neurotoxins of the three-finger toxin family, has been extensively studied (Ménez, 1985), the immunological properties of many toxin types remain largely unexplored. In the case of phospholipase A₂ (PLA₂) toxins, which are abundant and clinically relevant components of many snake venoms, immunological studies have mainly focused on those having potent neurotoxic activities, and have shown that elapid and viperid PLA₂s form two distinct antigenic classes (Kaiser et al., 1986; Henderson and Bieber, 1986; Middlebrook and Kaiser, 1989; Choumet et al., 1989, 1991, 1992; Mollier et al., 1989, 1990; Ćurin-Šerbek et al., 1991; Stiles and Middlebrook, 1991; Middlebrook, 1991; Basavarajappa et al., 1993; Alape-Girón et al., 1994; Cardoso et al., 2000; Demangel et al., 2000), in agreement with their evolutionary divergence into structural groups I and II, respectively (Schalloske and Dennis, 2006).

Within the venom PLA₂s from viperids (group II), a subdivision defined by the amino acid occupying position 49 exists: catalytically-active PLA₂s invariably present Asp49, whereas a subgroup of catalytically-inactive PLA₂ homologues most frequently present the substitution of this residue by Lys49 (Lomonte et al., 2003; Lomonte and Rangel, *in press*). All proteins of the Lys49 subgroup share the property of inducing skeletal muscle necrosis at the site of injection, therefore being classified as locally-acting myotoxins (Lomonte and Gutiérrez, 2011). Due to their abundance in the venoms of many viperids (Lomonte et al., 2009), and considering the clinical significance of myonecrosis in snakebite envenomings by such species (Cardoso et al., 1993; Otero et al., 2002), the Lys49 myotoxins represent important targets for neutralization by therapeutic antivenoms.

Some immunological aspects of the Lys49 myotoxins and their neutralization have been previously investigated using polyclonal and monoclonal antibodies (Lomonte et al., 1990b, 1992; Moura-da-Silva et al., 1991; Calderón and Lomonte, 1998; Angulo et al., 2001), but their immunorecognition at the molecular level has not been explored comprehensively. In the present study, a library of overlapping synthetic peptides spanning the complete sequence of myotoxin II from *Bothrops asper* was utilized to identify linear B-cell epitopes for the first time in a Lys49 PLA₂ homologue. Epitopes recognized by rabbit antibodies elicited by immunization with purified myotoxin II, or by horse antibodies present in therapeutic antivenoms, elicited by immunization with a mixture of crude venoms, were compared. As an additional aim of this immunological study, antibodies directed against an epitope located at the C-terminal region of myotoxin II,

known to be relevant for toxicity, were used as a probe to explore the possible dimeric assembly mode of this protein in solution.

2. Materials and methods

2.1. Toxin isolation

B. asper venom was a pool obtained from at least twenty adult specimens from the Caribbean region of Costa Rica, maintained at the serpentarium of Instituto Clodomiro Picado (University of Costa Rica). The venom was lyophilized and kept at -20°C . Myotoxin II was isolated from this venom by ion-exchange chromatography on CM-Sephadex C25 as described (Lomonte and Gutiérrez, 1989), followed by RP-HPLC on a C8 semi-preparative column (10×250 mm; Vydac) eluted at 2.0 mL/min with a 0–70% acetonitrile gradient containing 0.1% trifluoroacetic acid, during 30 min, on an Agilent 1200 instrument monitored at 215 nm. Toxin homogeneity was evaluated by MALDI-TOF mass spectrometry on an Applied Biosystems 4800-Plus instrument operated in positive linear mode, using sinapic acid as matrix, as previously described (Fernández et al., 2011).

2.2. Synthetic peptides

A library of 56 overlapping synthetic peptides (PepSets™), spanning the complete sequence of myotoxin II (P24506), was obtained from Mimotopes, Inc. (Minneapolis, USA). The offset of peptides was two amino acids. Peptides were biotinylated at the N-terminus, consisting of a tetrapeptide spacer arm (SGSG) followed by a dodecamer corresponding to each myotoxin II sequence segment, ending with an amidated C-terminus. This library was utilized in an enzyme-immunoassay for the linear epitope scanning of myotoxin II. The synthetic peptide KKYR-YYLKPLCKK, corresponding to the sequence 115–129 of myotoxin II (p115–129), coupled to diphteria toxoid, was utilized to raise rabbit antibodies to this region, as previously described (Calderón and Lomonte, 1998). Sequence numbering follows the scheme described by Renetseder et al. (1985).

2.3. Serum antibodies

Antiserum to myotoxin II was prepared by immunization of a white New Zealand female rabbit with the purified toxin, using complete Freund's adjuvant for priming (1 mg of toxin) and sodium alginate adjuvant for booster injections (0.25 mg) at three-week intervals, by i.m. route, during four months. Rabbit antiserum to synthetic peptide p115–129-diphteria toxoid was prepared similarly (Calderón and Lomonte, 1998). Antibodies to p115–129 were purified by affinity-chromatography on a column of myotoxin II immobilized onto CNBr-activated Sepharose 4B beads (GE Healthcare). Three batches of equine antivenom (Crotalidae polyvalent; 424LQ, 447LQ, and 466LQ), were provided by the Industrial Division of Instituto Clodomiro Picado. This antivenom consists of caprylic acid-purified, undigested immunoglobulins from the plasma

of horses hyperimmunized with a mixture of venoms from *B. asper*, *Crotalus simus*, and *Lachesis stenophrys* (Rojas et al., 1994).

2.4. Enzyme-immunoassay

Ninety-six well microplates (Nunc-Maxisorp®) were coated with streptavidin (0.5 µg/well) dissolved in 100 µL of 0.1 M Tris, 0.15 M NaCl buffer, pH 9.0, overnight at room temperature. After five washings with 0.12 M NaCl, 0.04 M sodium phosphate buffer, pH 7.2 (PBS), wells were blocked with bovine serum albumin (BSA; 1% in PBS) for 1 h, and then washed four times with PBS containing 0.05% Tween-20 (PBS-T). Biotinylated synthetic peptides (Section 2.2) were reconstituted in 40% acetonitrile/water at 10–15 mg/mL and stored as stock solutions at –20 °C. Peptide stocks were diluted 1:500 with PBS-T, and incubated into the streptavidin-coated wells for 1 h at room temperature. After four washings with PBS-T, either rabbit or horse antisera diluted 1:2000 in PBS-0.1% BSA were added and incubated for 2 h. After washing similarly, bound antibodies were detected by adding the corresponding anti-immunoglobulin/alkaline phosphatase conjugates (1:3000; Sigma-Aldrich) diluted in PBS-0.1% BSA for 1 h, followed by washing and final colour development with *p*-nitrophenylphosphate. Absorbances were recorded at 405 nm on a Multiskan FC-Thermo reader. Each peptide was assayed in duplicate wells. Non-immune sera of the corresponding animal species were utilized as negative controls to set the background values. Identification of a linear epitope was considered positive when an absorbance signal higher than two-fold the background value was recorded on at least two adjacent peptides.

2.5. Gel immunodiffusion

The ability of affinity-purified rabbit anti-p115-129 antibodies to immunoprecipitate myotoxin II was tested by gel immunodiffusion. Wells in 1% agarose-PBS were filled with toxin (50 µg) or antibodies (125 µg) in a volume of 30 µL, and allowed to diffuse overnight. The gel was exhaustively washed with PBS before staining the immunoprecipitate with amidoblack 10B, and finally destaining the background with 5% acetic acid.

2.6. Molecular visualization

The structure coordinates of myotoxin II (PDB code 1CLP; Arni et al., 1995) were employed to visualize the location of linear epitopes recognized by the anti-myotoxin II rabbit serum, or by the three samples of polyvalent equine antivenom. The crystal structure of a monoclonal IgG (1IGT) served to compare the molecular dimensions of an antibody relative to the epitope corresponding to the C-terminal region 115–129 of myotoxin II. Molecular images were prepared with RasWin v.2.7.5 (Biomolecular Structures Group, Glaxo Wellcome Research & Development) and DSViewer v.6.0 (Accelrys) software.

3. Results and discussion

In spite of the relevance of myotoxic PLA₂s from viperid venoms in the development of muscle tissue damage and potential sequelae in snakebites (Gutiérrez and Lomonte, 1995; Cardoso et al., 1993; Otero et al., 2002), insufficient work has been addressed at characterizing the antibody response to these antigens at the molecular level. Previous studies have shown that Lys49 myotoxins from different viperid species present significant immunochemical cross-reactivity (Lomonte et al., 1987, 1990a; Moura-da-Silva et al., 1991; Díaz et al., 1992; Angulo et al., 1997; Calderón and Lomonte, 1998), in agreement with the high amino acid sequence similarity among members of this toxin family. On this basis, present results obtained using *B. asper* myotoxin II might be useful to the immunological characterization of similar Lys49 myotoxins as well.

B-cell epitopes can be divided into two categories, conformational/discontinuous or linear/continuous (Worthington and Morgan, 1994). The former are more difficult to identify, being assembled by amino acids located at non-contiguous sites which are brought together by protein folding. In contrast, linear epitopes are more easily reproduced by currently available techniques for custom synthesis of peptides. The immunoassay design here utilized has the advantage of avoiding the direct binding of peptides to the solid-phase, thus minimizing their possible conformational alterations. Capturing of peptides in solution was possible by using an N-terminal biotin tag and solid-phase adsorbed streptavidin. In addition, a tetrapeptide spacer in each peptide (SGSG) provided an increased distance between the immobilized streptavidin and the dodecameric antigenic probe, reducing steric hindrance effects in the immunoassay. With this design, several linear epitopes were identified in myotoxin II, when probed against rabbit (Fig. 1) and horse (Fig. 2) hyperimmune sera. Some of the epitopes were shared between the two species, whereas others were unique. A gradient of immunodominance among the different epitopes was also observed, as implied from the variable intensities of the immunoassay signals obtained. Some of the linear epitopes identified were weak (VI, VII, VIII), especially in the case of the equine antivenoms (Fig. 2). Strongest reactivity in the rabbit serum was observed against epitopes III and IV (Fig. 1), whereas the three equine antivenoms had in common a marked preference for epitope II (Fig. 2), which was also significantly recognized by the rabbit antibodies. Differences in epitope recognition were observed not only between the two species, but also within the three batches of horse-derived antivenom. Such variability is expected on the basis of the different genetic constitution of individuals within a species, especially at their MHC loci (Benacerraf, 1981). The location of the identified epitopes for both rabbit and horse antibodies in the three-dimensional structure of a myotoxin II monomer is comparatively represented in Fig. 3.

Depending on the functional consequences of antibody binding to a toxin, epitopes can be classified as neutralizing or non-neutralizing. In the case of myotoxin II, previous

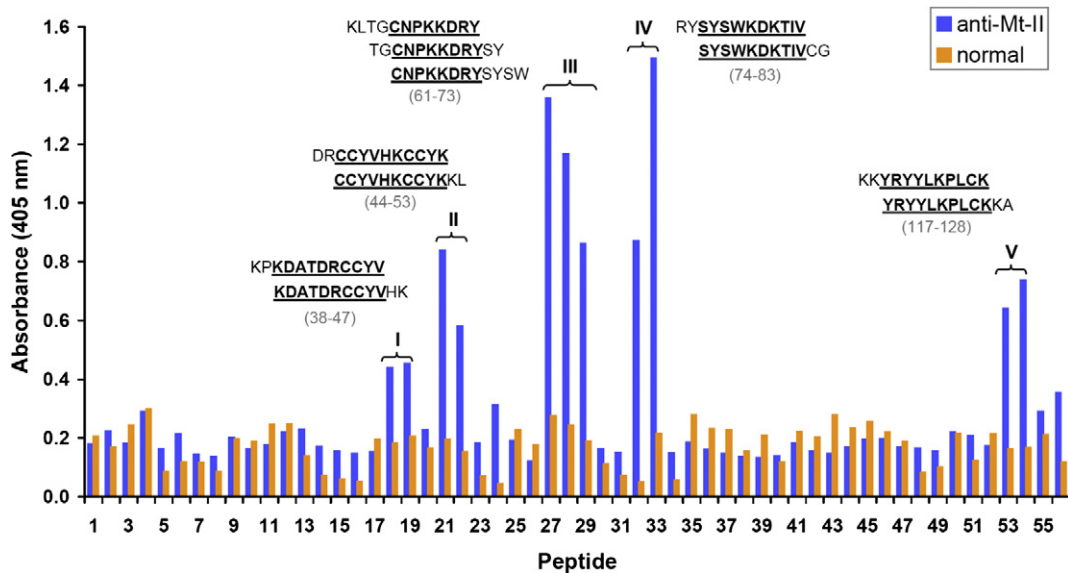


Fig. 1. Linear epitopes recognized by rabbit serum antibodies to *Bothrops asper* myotoxin II. A library of 56 biotinylated synthetic peptides of myotoxin II (dodecamers, with an overlapping offset of two), bound to streptavidin-coated 96-well plates, was incubated with immune rabbit serum (anti-Mt-II) or non-immune rabbit serum (normal). Bound antibodies were detected colorimetrically by enzyme-immunoassay, as described in Materials and Methods. Absorbance signals higher than two-fold the value of normal serum background controls, and occurring in at least two contiguous peptides, were considered as linear epitopes (labeled in roman numerals). The amino acid sequences corresponding to the recognized peptides are indicated, with shared sequences in boldface and underlined. Amino acid sequence numbering follows Renetseder et al. (1985).

studies have shown that antibodies specifically directed to the C-terminal region 115–129 inhibit its toxic actions (Calderón and Lomonte, 1998). Thus, the epitope here identified as “V”, spanning residues 117–128, is of functional interest. Although the rabbit serum to myotoxin II clearly recognized it, only one out of the three batches of equine antivenom contained antibodies to this epitope. This finding is in agreement with the earlier observation that, although antibodies to this site can be raised by immunization with a synthetic peptide, this particular region is not strongly immunodominant in the antibody response against the complete toxin, or against crude venoms containing the toxin. A previous screening of different batches of polyvalent-Crotalidae equine antivenom showed that only seldom they contained antibodies against region 115–129 of myotoxin II (Calderón and Lomonte, 1998), essentially corresponding to epitope V. Moreover, immunization of mice with intact myotoxin II did not induce significant levels of antibodies to peptide 115–129, whereas a strong antibody response to this epitope was obtained by immunization of mice with the corresponding synthetic peptide (Calderón and Lomonte, 1999).

Excluding epitope V, it remains to be determined if other epitopes identified in the present study lead to toxin neutralization or not. This will require generating specific antibodies against each of these epitopes and testing their neutralizing ability. Although the myotoxic PLA₂s are relatively small proteins in comparison to antibodies, previous investigations using a set of seven mouse monoclonal antibodies demonstrated the existence of both neutralizing and non-neutralizing epitopes (Lomonte and Kahan, 1988;

Lomonte et al., 1992). However, the epitopes recognized by these monoclonal antibodies were of the conformational type, and could not be identified by using linear synthetic peptide strategies.

The molecular characterization of epitopes on toxins encloses potential benefits, both from an applied and a basic perspective. Neutralizing epitopes have been used as synthetic immunogens to raise antisera against a variety of toxin types, including snake venom metalloproteinases (Ferreira et al., 2006; Cardoso et al., 2009; de Avila et al., 2011) and PLA₂s (Demangel et al., 2000), scorpion neurotoxins (Calderon-Aranda et al., 1999; Gazarian et al., 2005), or spider dermonecrotic toxins (Dias-Lopes et al., 2010), among other examples. In the case of myotoxin II, immunization of mice with its C-terminal synthetic peptide 115–129 induced antibodies that prevented by 40% the muscle damage induced by an experimental toxin challenge, in comparison to non-immunized animals (Calderón and Lomonte, 1999). Nevertheless, protection from myonecrosis in such model was still lower than that obtained by immunization with the intact toxin. The present observation that linear epitopes III and IV (in rabbit serum) and II (in equine antivenoms) display stronger recognition by antibodies than other toxin regions, prompts for an exploration of their potential as immunogens in future studies, aiming to enhance the levels of anti-myotoxin antibodies during equine antivenom production. In this regard, it is relevant to note that antivenomic analyses based on HPLC-immunodepletion and immunoblotting techniques have recently disclosed that the antibody response to snake venom PLA₂s is sometimes insufficient, suggesting that

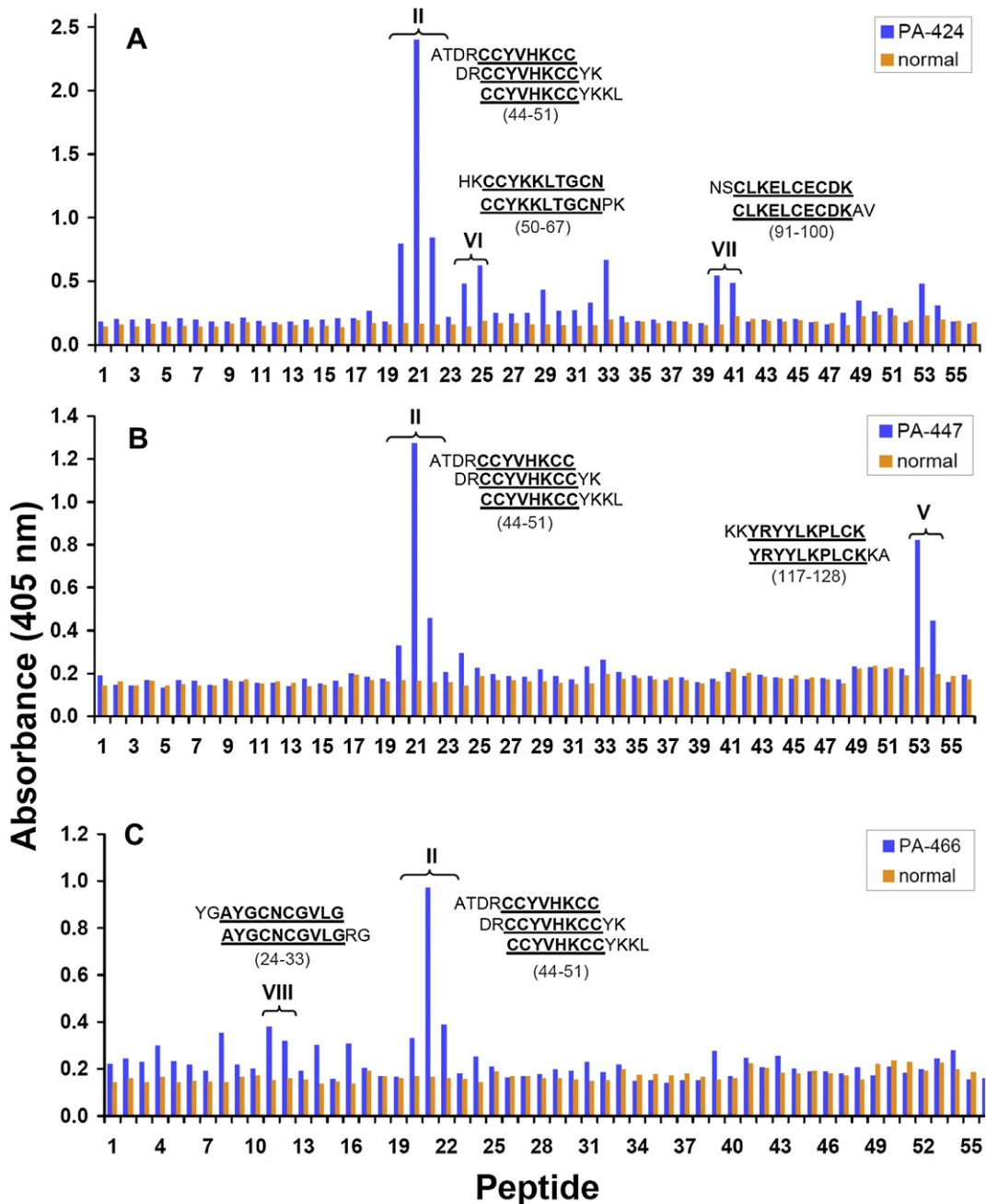


Fig. 2. Linear epitopes of *Bothrops asper* myotoxin II recognized by equine antibodies from three batches of therapeutic polyvalent (Crotalidae) antivenom. A library of 56 biotinylated synthetic peptides of myotoxin II (dodecamers, with an overlapping offset of two), bound to streptavidin-coated 96-well plates, was incubated with three equine polyvalent antivenoms (A, PA-424; B, PA-447; C, PA-466) or non-immune equine immunoglobulins (normal). Bound antibodies were detected colorimetrically by enzyme-immunoassay, as described in Materials and Methods. Absorbance signals higher than two-fold the value of the corresponding normal background controls, and occurring in at least two contiguous peptides, were considered as linear epitopes (labeled in roman numerals, and continuing the numbering in Fig. 1). The amino acid sequences corresponding to the recognized peptides are indicated, with the shared sequences in boldface and underlined. Amino acid sequence numbering follows Renetseder et al. (1985).

the immunogenicity of these relatively small proteins (15 kDa) may be limited (Lomonte et al., 2008; Antúnez et al., 2010; Fernández et al., 2011; Calvete et al., 2011). Similarly, an evaluation of anti-myotoxin antibodies present in various therapeutic antivenoms by means of

enzyme-immunoassay revealed in some cases unexpectedly low titers (Lomonte et al., 1991). Manipulation of the immune response during antivenom production, for example by using synthetic peptides that represent neutralizing toxin epitopes, or recombinant DNA strings

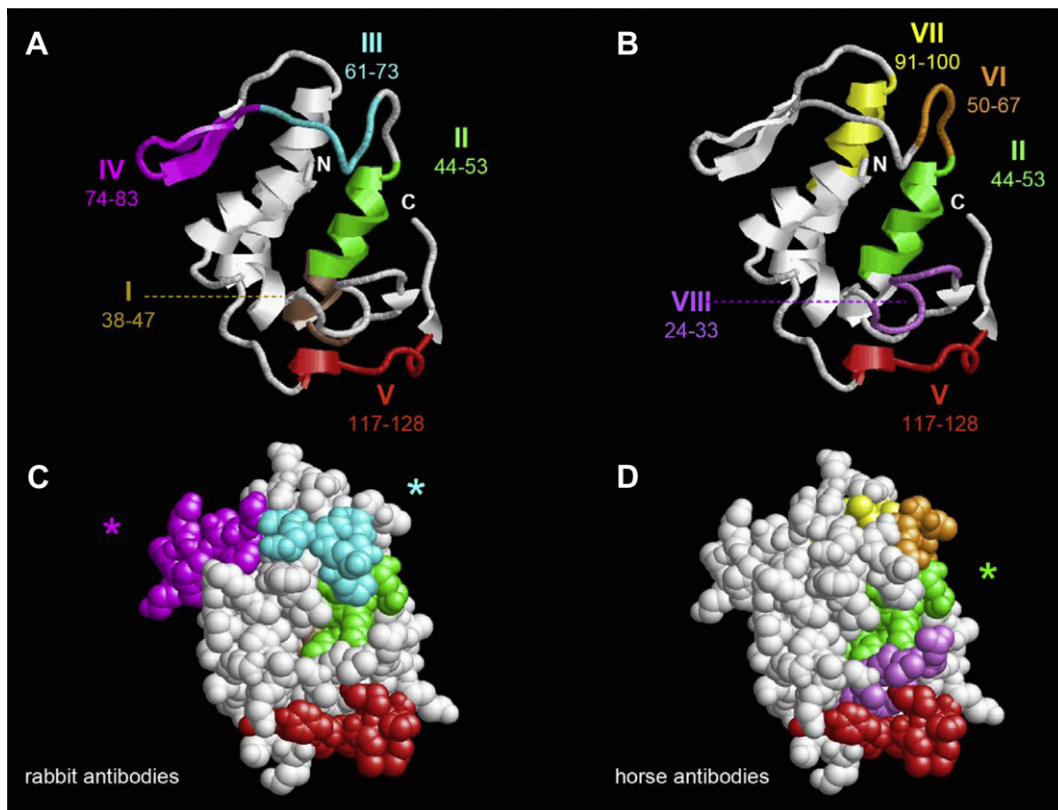


Fig. 3. Mapping of linear epitopes recognized by rabbit (A,C) and horse (B, D) antibodies on *Bothrops asper* myotoxin II. The three-dimensional structure of a myotoxin II monomer (PDB code 1CLP; Arni et al., 1995) is represented in ribbons (top) or in space-filling view (bottom). The location of linear epitopes identified from data in Fig. 1 (rabbit antibodies) and Fig. 2 (horse antibodies) is shown with different colours on a grey backbone, and indicating the sequence numbering of the segments. Epitopes with shared recognition by antibodies from both species are coloured identically. In panels C and D, epitopes providing the strongest signals in the analyses from Fig. 1 (III and IV) and 2 (II) are indicated by asterisks. Images were prepared with RasWin v.2.7.5. For interpretation of colours the reader is referred to the web version of the article.

coding for these (Wagstaff et al., 2006), could prove useful to enhance the efficacy of these products against particularly relevant toxins which may be naturally weak as immunogens.

From a basic point of view, epitope mapping offers some possibilities to study the structure–function relationships of toxins. Lys49 myotoxins exist mainly as homodimers in solution (Lomonte and Rangel, in press), but two contrasting modes of dimerization have been described by crystallographic analyses, referred to as “conventional” (Arni et al., 1995; da Silva Giotto et al., 1998) and “alternative” (dos Santos et al., 2009) dimers (Fig. 4). It is still controversial if both types of quaternary structural arrangements can occur in these toxins, or if only one of these models is the correctly deduced form. Myotoxin II was originally described as a “conventional” dimeric assembly (Arni et al., 1995), but several other related Lys49 myotoxins have been more recently reported as “alternative” dimers (dos Santos et al., 2009, 2010). For this reason, as a second aim of the present study, an exploration of the spatial availability of the region corresponding to epitope V of myotoxin II was

attempted, using the affinity-purified rabbit antibodies to peptide 115–129 in a gel immunodiffusion assay. As shown in Fig. 4, these site-specific antibodies were capable of precipitating myotoxin II, therefore implying the simultaneous availability of the two copies of epitope V (in the toxin dimer), to the binding by two independent antibody molecules needed to grow multimolecular complexes large enough to precipitate. Due to the larger size of antibodies relative to the toxin (proportionally represented in Fig. 4), it would seem difficult that multivalent binding of the toxin may have occurred if its structure corresponded to the “alternative” dimeric assembly, due to the steric hindrance imposed by the antibody paratope in the vicinity of its recognized epitope (Fig. 4). In contrast, the distance between the two copies of epitope V in the “conventional” dimer model would seem compatible with the formation of multimeric antigen–antibody complexes. Although this experiment only provides an indirect structural evidence, and as such should be interpreted with caution, it would be in agreement with the originally proposed “conventional” mode of dimerization for myotoxin II.

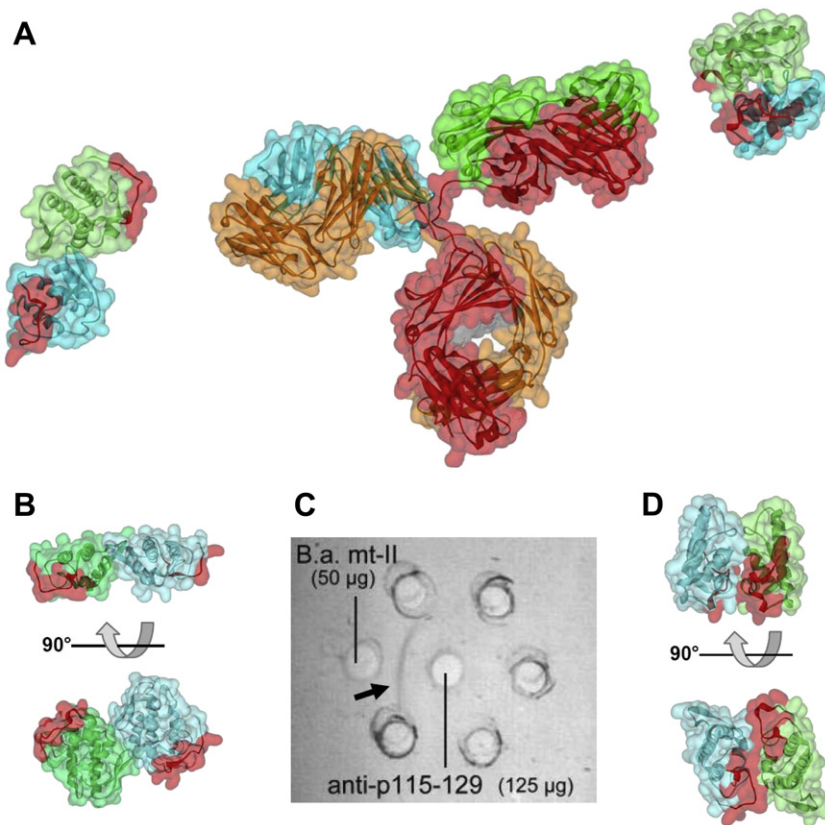


Fig. 4. Implications of the spatial availability of epitope V for the dimeric assembly of *Bothrops asper* myotoxin II, as inferred from immunoprecipitation. Rabbit antibodies to a synthetic peptide representing the sequence 115–129 of myotoxin II were raised by immunization with diphtheria toxoid-conjugated peptide. The obtained antibodies were purified by affinity-chromatography on a column of Sepharose 4B-myotoxin II, and tested by agarose gel double immunodiffusion against this toxin. As shown in C, antibodies to p115–129 formed an immunoprecipitation line (arrow), implying the formation of multimolecular complexes between the divalent IgG molecules and the dimeric toxin. Two possible modes of dimerization of myotoxin II are represented in panels B (“conventional” dimer) and D (“alternative” dimer), respectively, showing the location of the sequence 115–129 in red, corresponding to epitope V (see Figs. 1–3). Panel A compares the scaled molecular dimensions of an IgG (PDB code 1IGT) relative to those of myotoxin II in a conventional (left) or an alternative (right) dimeric assembly. Notice the spatial separation of the two repeats of epitope V (red) in the conventional dimeric assembly, which could allow the simultaneous binding of two IgG molecules needed for immunoprecipitation, in contrast to the spatial restraints imposed by the alternative dimeric assembly, where the proximity of the two repeats of epitope V (red) would be expected to cause steric hindrance after the binding of a single antibody paratope, thereby precluding the growth of complexes. Molecular images were prepared with DSVIEWER 6.0, shown in ribbons representation with semi-transparent molecular surfaces. For interpretation of colours the reader is referred to the web version of the article.

Conflict of interest statement

The author declares that there are no conflicts of interest.

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