

Solving the microheterogeneity of *Bothrops asper* myotoxin-II by high-resolution mass spectrometry: Insights into C-terminal region variability in Lys49-phospholipase A₂ homologs

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ABSTRACT

Myotoxin-II, a phospholipase A₂ (PLA₂)-like protein found in *Bothrops asper* venom, causes rapid necrosis of muscle fibers in spite of lacking enzymatic activity. This toxic action maps to its C-terminal region, within a segment known as “115–129” (consensus numbering) that displays a combination of cationic and hydrophobic amino acids, capable of destabilizing membranes. Although myotoxin-II is found in *B. asper* from both the Caribbean and Pacific regions of Costa Rica, this work shows that in the latter, position 124 is occupied by phenylalanine, instead of leucine reported for the Caribbean variant (UniProt P24605), thus solving the ambiguity described in the original sequencing of this toxin. A comparative inspection of sequences in the C-terminal region of 70 PLA₂-like proteins showed that, with few exceptions, position 124 is occupied by either leucine or phenylalanine with equal frequencies. In line with earlier observations on primary and three-dimensional structural data, this comparison supports the notion that the disruptive mechanism of PLA₂-like myotoxins toward membranes is not dependent on a fixed amino acid sequence motif across members of this subfamily, but instead on a spatial array of physicochemical properties which can be provided by variable combinations of cationic and hydrophobic residues. This plasticity bears resemblance to features of many antimicrobial peptides acting upon membranes. From a practical point of view, it is recommended to define the identity of the many isoforms of PLA₂s and PLA₂-like proteins found in viperid venoms by relying on the accurate determination of their intact mass, as these proteins are not known to present post-translational modifications.

1. Introduction

Necrotic muscle damage is a potential consequence of snakebite envenomings that may lead to permanent tissue loss and disability (Warrell, 2010; Abubakar et al., 2010; Means et al., 2017). Venom proteins targeting skeletal muscle fibers to cause necrosis – myotoxins – belong to three structural types: “three-finger” cytotoxins, of ~60 amino acids, typically found in elapid snakes; “small” myotoxins, of 42 amino acids, found in various rattlesnakes; and phospholipases A₂ (PLA₂s), of ~120 amino acids, generally widespread among both elapid and viperid snake species (Mebs and Ownby, 1990; Lomonte and Rangel, 2012). Of these types, PLA₂s have been more intensively studied to decipher their mechanisms of toxicity (Harris and Cullen, 1990; Ohno et al., 2003; Montecucco et al., 2008; dos Santos et al., 2009; Lomonte and Gutiérrez, 2011; Lomonte and Križaj, 2021).

The muscle damaging mechanism of snake venom PLA₂ myotoxins is

only partially understood, but known to depend on their ability to enzymatically hydrolyze membrane phospholipids, generating free fatty acids and lysophospholipids, and ultimately destabilizing its ion permeability and integrity (Cintra-Francischinelli et al., 2009; Fernández et al., 2013). Nevertheless, a subgroup of PLA₂-like proteins exists in the venoms of many viperids, which in spite of lacking enzymatic activity can induce myonecrosis, therefore using a different mechanism than true PLA₂s (Lomonte and Rangel, 2012; Fernández et al., 2013). Since their discovery (Maraganore et al., 1984) more than 80 of these so-called “Lys49 PLA₂-like proteins” or “Lys49 PLA₂ homologs” have been described, often being present together with enzymatically active “Asp49 PLA₂ myotoxins” counterparts in the same venom, and showing synergism in their toxic actions (Cintra-Francischinelli et al., 2009; Mora-Obando et al., 2014; Bustillo et al., 2019). PLA₂-like myotoxins have the ability to permeabilize membranes in the absence of phospholipid hydrolysis (Rufini et al., 1992; de Oliveira et al., 2009;

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Fernández et al., 2013), causing a massive influx of calcium, among various alterations that lead to hypercontraction of myofibers and rapid necrotic cell death (Montecucco et al., 2008).

The membrane permeabilizing mechanism of Lys49 myotoxins involves the participation of amino acids located near the C-terminus. Short synthetic peptides corresponding to the sequence “115–129” (consensus numbering of Renetseder et al., 1985) of some Lys49 myotoxins can mimic their toxicity (Lomonte et al., 1994, 2003a, 2003b; Páramo et al., 1998; Núñez et al., 2001; Costa et al., 2008; Gebrim et al., 2009; Cintra-Francischinelli et al., 2010; Almeida et al., 2018), and site-directed mutations within this C-terminal region affect their toxic actions (Ward et al., 2002; Chioato et al., 2002, 2007). Furthermore, crystallographic studies on several Lys49 myotoxins support the notion that some cationic amino acid residues at the C-terminal region promote membrane docking, while others, of hydrophobic or aromatic nature, are involved in bilayer destabilization through allosteric reorientations triggered by ligand binding to their hydrophobic channel (Ambrosio et al., 2005; Fernandes et al., 2014; Borges et al., 2017).

Altogether, evidence gathered thus far indicates that characterizing the C-terminal region of Lys49 myotoxins is relevant to advance understanding of their toxic mechanism. However, a limitation that may arise during purification of these toxins, concerns heterogeneity: venoms often contain a complex mixture of several myotoxin variants, i. e., isoforms encoded by distinct genes evolved by duplication and divergence (Nakashima et al., 1995). Isoforms present slight variations of their amino acid sequences, and consequently this

microheterogeneity may introduce uncertainties in interpreting the subtle structure/function relationships of these myotoxic proteins.

One example of microheterogeneity is represented by *Bothrops asper* myotoxin-II. After its initial description (Lomonte and Gutiérrez, 1989), Francis et al. (1991) reported its amino acid sequence of 121 residues as presenting an unresolved ambiguity at position 114 (or 124 in the consensus numbering), being either leucine (Leu) or phenylalanine (Phe) (Fig. 1A). Three decades ago, its sequence was submitted to UniProt with the code P24605, reported to present Leu. With the benefits of currently available high-resolution mass spectrometry, the present study re-examined *B. asper* myotoxin-II in venoms obtained from specimens of the Caribbean and Pacific regions of Costa Rica, respectively, known to present significant overall protein compositional differences (Alape-Girón et al., 2008), aiming to resolve its microheterogeneity. In addition, amino acid sequence variability in the C-terminal region of all PLA₂-like proteins currently available was examined, aiming to gain further insights into its proposed role in toxicity.

2. Materials and methods

2.1. Venoms

Venoms pooled from more than 20 specimens of *B. asper* from the Caribbean (BaC) or the Pacific (BaP) regions of Costa Rica, respectively, kept in captivity at the Serpentarium of Instituto Clodomiro Picado, were lyophilized and kept at -20°C until use.

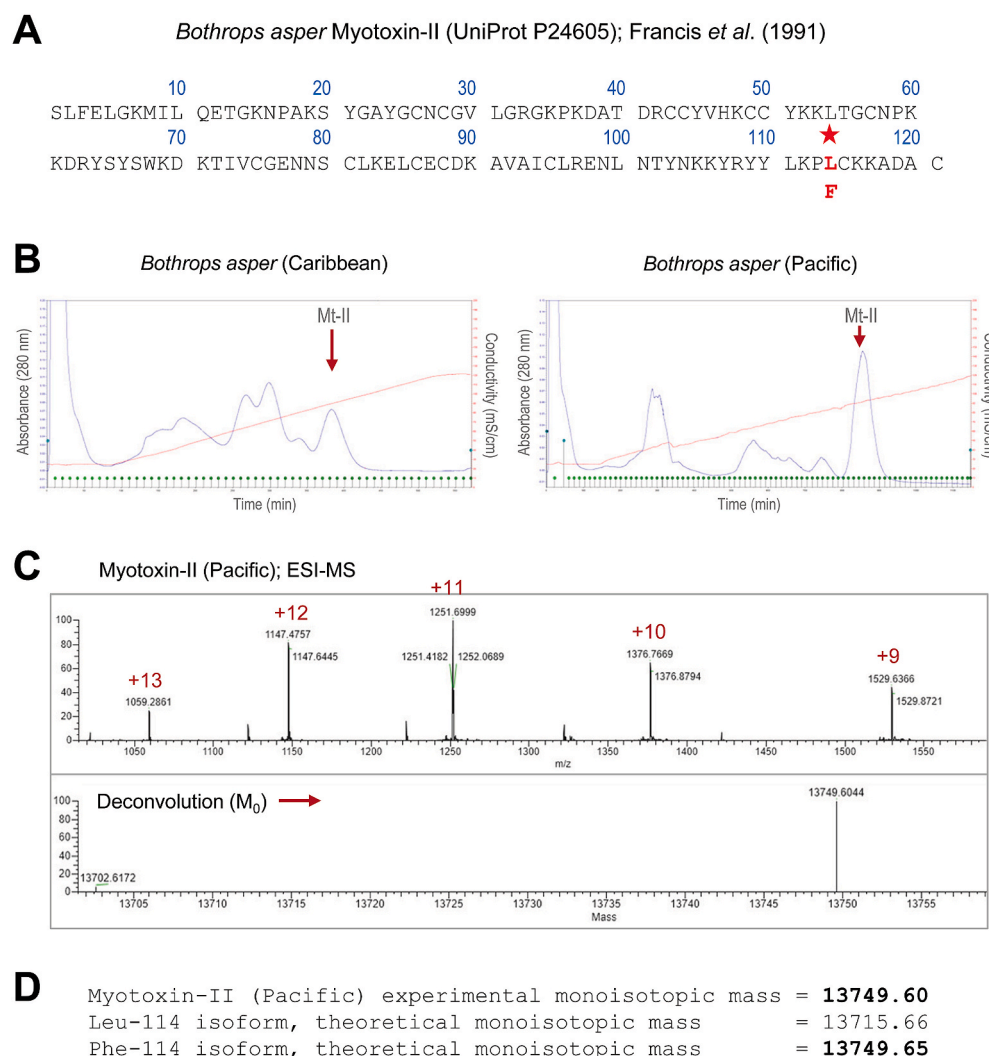


Fig. 1. (A) The amino acid sequence ambiguity reported for *Bothrops asper* myotoxin-II (UniProt P24605) at position 114 (L/F) is highlighted in red with a star. (B) Cation-exchange chromatography of *B. asper* venoms from the Caribbean (left) or Pacific (right) regions of Costa Rica. The peak corresponding to myotoxin-II is indicated with a red arrow. (C) High-resolution intact monoisotopic mass determination for myotoxin-II from the Pacific region. The multiply-charged ion series is shown in the top spectrum, and its deconvolution at the bottom, indicating the monoisotopic mass (M_0). (D) Matching of the experimentally determined monoisotopic mass of myotoxin-II (Pacific) with the theoretically calculated value when considering phenylalanine at position 114. For interpretation of colors in this figure the reader is referred to the online version of the article.

2.2. Cation-exchange chromatography

Venom samples of 100 mg (BaC or BaP) were dissolved in 10 mL of 0.05 M Tris, 0.1 M KCl, pH 7.0 buffer, and centrifuged. The supernatant was applied to a column of CM-Sephadex® C-25 (25 × 2.5 cm; Cytiva) equilibrated with the same buffer, and separated at 0.5 mL/min using a BioLogic® LP chromatography system (Bio-Rad) monitored at 280 nm. After the initial peak of unbound acidic proteins returned to baseline, elution of basic proteins was performed with a linear gradient toward 0.75 M KCl, in the same buffer, as previously described (Lomonte and Gutiérrez, 1989).

2.3. RP-HPLC

Venom samples of 2 mg were dissolved in 200 µL of water containing 0.1% trifluoroacetic acid (solution A) and centrifuged. The supernatant was applied to a reverse-phase column (C₁₈, 250 × 4.6 mm, 5 µm particle; Phenomenex) equilibrated with the same solution, and separated at 1 mL/min using an Agilent 1220 chromatography system monitored at 215 nm. Elution was performed with a gradient toward acetonitrile containing 0.1% trifluoroacetic acid (solution B), as follows: 0% B for 5 min, 0–15% B in 10 min, 15–45% B in 60 min, 45–70% B in 10 min, and 70% B for 9 min (Lomonte and Calvete, 2017). Fractions of interest were manually collected and dried by vacuum centrifugation (Vacufuge®; Eppendorf) for further mass spectrometry analyses.

2.4. Mass spectrometry

Intact mass of myotoxin isoforms obtained from the RP-HPLC fractionation of BaC and BaP venoms was determined by direct infusion ESI-MS in a Q-Exactive Plus® instrument (Thermo). Proteins were dissolved in 50% acetonitrile/water containing 0.1% formic acid at 50–100 µg/mL, and infused at 5 µL/min into a HESI source, to acquire a full MS scan in the 800–2000 m/z range, at resolution of 140,000 (at m/z 240), in positive mode. The acquired MS spectra of the multiply-charged ion series were deconvoluted using Freestyle® v.1.5 (Thermo) to obtain monoisotopic masses.

To further validate the presence of Phe114 in myotoxin-II obtained from BaP venom, the protein was subjected to SDS-PAGE under reducing conditions, stained with Coomassie blue, excised and in-gel digested overnight with sequencing-grade trypsin after reduction with 10 mM dithiothreitol and alkylation with 50 mM iodoacetamide, using an automated workstation (Intavis). The resulting peptides were dried, redissolved in water with 0.1% formic acid, and submitted to nano-LC-MS/MS. Ten µL of digest were loaded on a C₁₈ trap column (75 µm × 2 cm, 3 µm particle; PepMap®, Thermo), washed with 0.1% formic acid (solution A), and separated at 200 nL/min with a 3 µm particle, 15 cm × 75 µm C₁₈ Easy-spray® analytical column using a nano-Easy® 1200 chromatograph (Thermo). A gradient from 0.1% formic acid (solution A) to 80% acetonitrile with 0.1% formic acid (solution B) was developed: 1–5% B in 1 min, 5–26% B in 25 min, 26–79% B in 4 min, 79–99% B in 1 min, and 99% B in 4 min, for a total time of 35 min. MS spectra were acquired in positive mode at 1.9 kV, with a capillary temperature of 200 °C, using 1 µscan at 400–1600 m/z, maximum injection time of 100 msec, AGC target of 3×10^6 , and orbitrap resolution of 70,000. The top 10 ions with 2–5 positive charges were fragmented with AGC target of 1×10^5 , maximum injection time of 110 msec, resolution 17,500, loop count 10, isolation window of 1.4 m/z, and a dynamic exclusion time of 5 s. MS/MS spectra were processed for peptide matching with protein sequences contained in the UniProt/SwissProt database for Serpentes, as well as with the sequence of myotoxin-II (access code P24605) after introducing the Phe substitution in the FASTA file, using Peaks X® (Bioinformatics Solutions). Cysteine carbamidomethylation was set as a fixed modification, while deamidation of asparagine or glutamine and methionine oxidation were set as variable modifications, allowing up to 2 missed cleavages by trypsin. Parameters for match

acceptance were set to FDR < 1%, and –10lgP protein score ≥ 30.

2.5. Amino acid sequence alignment of PLA₂-like proteins

BLAST (<https://blast.ncbi.nlm.nih.gov>) was used to retrieve the 250 amino acid sequences with highest similarity to *B. asper* myotoxin-II (P24605) available in Uniprot, all of them corresponding to PLA₂s or PLA₂-like proteins from snake venoms. From these, all PLA₂ sequences with aspartic acid at position 49 were deleted, as well as few duplicate entries and partial sequences, leaving 101 entries corresponding to “non-Asp49” (mostly Lys49, but also few Ser49, Asn49, and Arg49) proteins. These were aligned by using the Muscle algorithm in MEGA (Kumar et al., 2018). Then, only the C-terminal regions (106–125 of this alignment) were compared, and entries corresponding to isoforms from a single species having identical amino acid sequence in this region were manually deleted, to leave only one representative in the final alignment of 70 sequences. Positions 106–119 of the alignment correspond to the consensus numbering “115–129” of Renetseder et al. (1985) generally used in the literature and databases. The final alignment was used to generate a WebLogo plot to represent amino acid sequence variability among all the “non-Asp49” proteins, using WebLogo 3 (weblogo.threepl.usone.com). The three-dimensional structure of a monomer of *B. asper* myotoxin-II (PDB code 1CLP) was used as a visual guide to the spatial organization of the “115–129” region in this protein group. This protein presents a homodimeric structure in crystallographic studies (Arni et al., 1995; Murakami et al., 2005), but has been found as a monomer in solution (Soares et al., 2022).

3. Results

Fractionation of *B. asper* venom (Caribbean region; BaC) by cation-exchange chromatography showed the pattern originally reported for the isolation of myotoxin-II (Lomonte and Gutiérrez, 1989), which corresponds to the last eluting (most basic) protein peak (Fig. 1B, left). In the case of venom from Pacific region specimens (BaP), this chromatographic procedure resulted in a more prominent and better resolved peak of myotoxin-II (Fig. 1B, right). Since the original characterization and amino acid sequencing of myotoxin-II (P24605) was made using BaC venom, the counterpart protein isolated from BaP venom was investigated by mass spectrometry. As shown in Fig. 1C, the high-resolution MS spectrum of intact myotoxin-II determined a monoisotopic mass of 13,749.60 Da. This value is in agreement with the theoretical mass expected for the amino acid sequence P24605 when calculated with Phe114, instead of Leu114 (Fig. 1D). The presence of Phe114 in myotoxin-II isolated from BaP venom was further confirmed by trypsin digestion and nano-LC-MS/MS, as shown in Fig. 2.

The complexity of myotoxin isoforms in BaC and BaP venoms was further explored by examination of their RP-HPLC patterns in combination with intact mass spectrometry. Myotoxin-II and closely related variants elute in the central region of the chromatographic profile, between approximately 50–56 min (dotted boxes in Fig. 3A and Fig. 3B). In this region, BaC venom showed three prominent peaks of roughly similar heights (Fig. 3A), although at least two poorly resolved peak shoulders were additionally evidenced (Fig. 3B). In comparison, the equivalent region in the BaP profile showed a marked difference in the proportions of the three main peaks, with the central one being predominant (Fig. 3D), and no additional peak shoulders were evidenced (Fig. 3C).

Mass spectrometry of the predominant central peak of BaP venom revealed a homogeneous protein of 13,750.5 Da (Fig. 3C), corresponding within less than 1 Da error to the intact monoisotopic mass of the Phe114 isoform of myotoxin-II. On the other hand, the equivalent central peak of BaC venom presented a predominant protein of 13,716.5 Da (Fig. 3B), in agreement with the theoretical intact mass calculated for the Leu114 isoform of myotoxin-II. In addition, this BaC venom peak showed lower proportions of two other proteins of 13,750.5 (Phe114

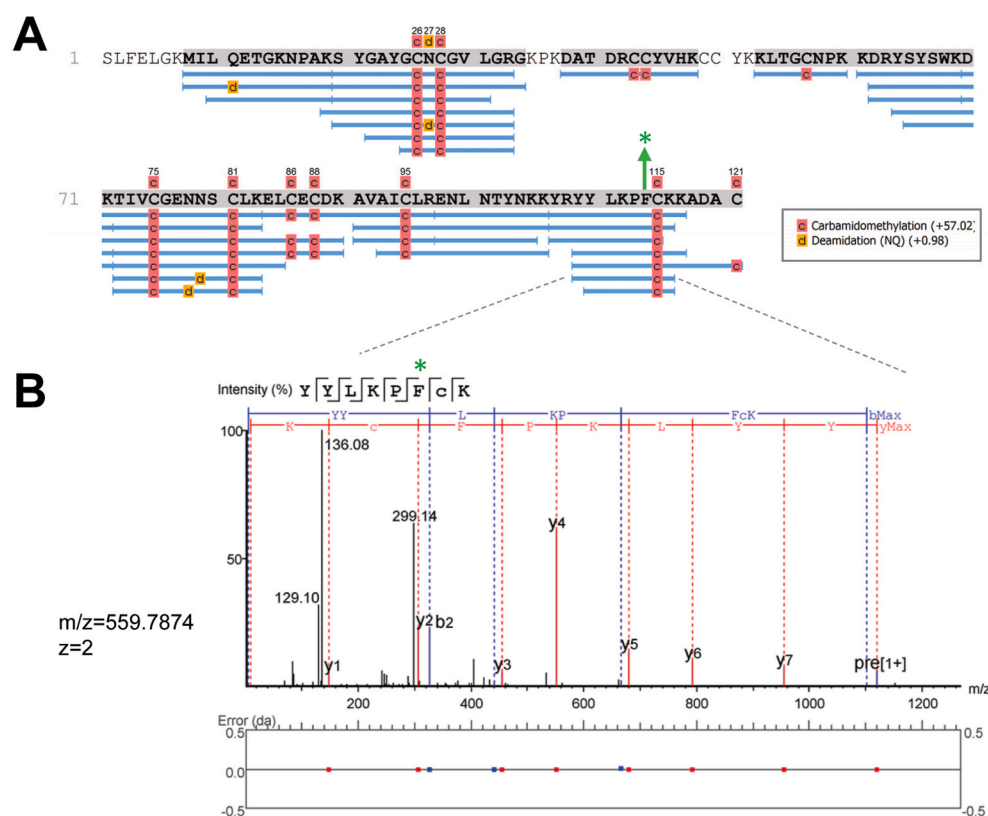


Fig. 2. (A) Bottom-up analysis of peptides obtained by the digestion of *Bothrops asper* myotoxin-II from the Pacific region with trypsin, followed by nano-LC-MS/MS. Phenylalanine at position 114 is indicated by a green asterisk and arrow. Light blue lines represent peptides matching the UniProt sequence P24605 edited to present Phe114 in the database used for the Peaks X® search. (B) MS/MS fragmentation spectrum of a peptide (m/z 559.7874, $z = 2^+$) covering the Phe114 position, with error in mass measurements shown in ppm at the bottom. For interpretation of colors in this figure the reader is referred to the online version of the article.

variant) and 13830.5 Da. Overall, the BaC venom profile showed a higher complexity of myotoxin variants in the RP-HPLC resolved peaks, when compared to the BaP profile, and none of the BaC peaks appeared to be homogeneous in terms of the intact masses found (Fig. 3B).

Finally, an examination of the Phe/Leu heterogeneity in the C-terminal region of all currently available “non-Asp49” PLA₂-like proteins from snake venoms was performed (Fig. 4). This comparison evidenced that the position preceding a conserved cysteine (position 116 in the alignment of Fig. 4, equivalent to 124 in the consensus numbering) is occupied by either Leu or Phe in roughly an equal number of the proteins known to date, and that these two amino acids are predominant. A smaller group of toxins from three *Vipera* species (Old World) and four *Agkistrodon* subspecies (New World) instead present lysine at this position, although it is immediately preceded by Phe in the former group, or either by Phe/Leu in the latter (Fig. 4). Another feature can be pointed out at position 115 of the alignment, preceding the Leu/Phe microheterogeneity here analyzed, where the majority of proteins from New World viperids present proline, in contrast to those of Old World species. The sequence variability described in Fig. 4 was represented as a WebLogo plot in Fig. 5B, and related to the spatial arrangement of amino acids 106–125 (i.e., the “115–129” region) in the three-dimensional structure of *B. asper* myotoxin-II from the Caribbean (PDB code 1CLP; Arni et al., 1995) in Fig. 5A. The plot shows that cationic amino acid residues in this region, usually about six out of the thirteen positions, as well as two tyrosines, are more conserved in comparison to other positions occupied by hydrophobic residues.

4. Discussion

4.1. *B. asper* myotoxin-II differs in venom from Caribbean and Pacific regions

PLA₂s and PLA₂-like myotoxins are major culprits of the local muscle damage that develops in envenomings by many viperids, and this has

inspired studies to understand their mode of action, neutralization by antibodies, or inhibition by small compounds. *B. asper* myotoxin-II, a Lys49 protein, was first isolated from specimens of the Caribbean region of Costa Rica (Lomonte and Gutiérrez, 1989), and subsequently found also in *B. asper* from the Pacific region. Therefore, a number of studies have investigated this myotoxin obtained indistinctly from either of these two geographic origins, but more often from the Pacific in recent years, owing to the higher availability of the venom.

The present work re-examined myotoxin-II in venoms obtained from both regions to investigate the amino acid sequence ambiguity that was reported at position 114 (Francis et al., 1991). High-resolution mass spectrometry demonstrated that myotoxin-II from BaP presents Phe, instead of Leu reported in Uniprot entry P24605. The originally reported ambiguity is now explained by observing that myotoxin-II from BaC consists predominantly of the Leu114 variant (13,716 Da), whereas its counterpart from BaP corresponds to the Phe114 variant (13,750 Da). In addition, the present chromatographic and mass spectrometry combined approach showed that BaP venom is a more convenient source than BaC for the isolation of myotoxin-II, providing not only higher homogeneity but also higher yields.

4.2. Intact protein mass as a key toxin identifier to clarify structure-function studies

The presence of multiple isoforms appears to be a common finding for snake venom PLA₂s and PLA₂-like myotoxins (Soares et al., 1998; Chijiwa et al., 2003; Ikeda et al., 2010; Salvador et al., 2021), even when venom is sampled from individual snakes as demonstrated in *B. asper* (Lomonte and Carmona, 1992). This makes protein purification to homogeneity sometimes difficult and more likely to introduce ambiguities due to the microheterogeneity of variants (Francis et al., 1991; Borges et al., 2021, 2022; Salvador et al., 2021). Therefore, it is suggested that a description of the intact protein mass when new myotoxins are characterized, and its verification when following previously described

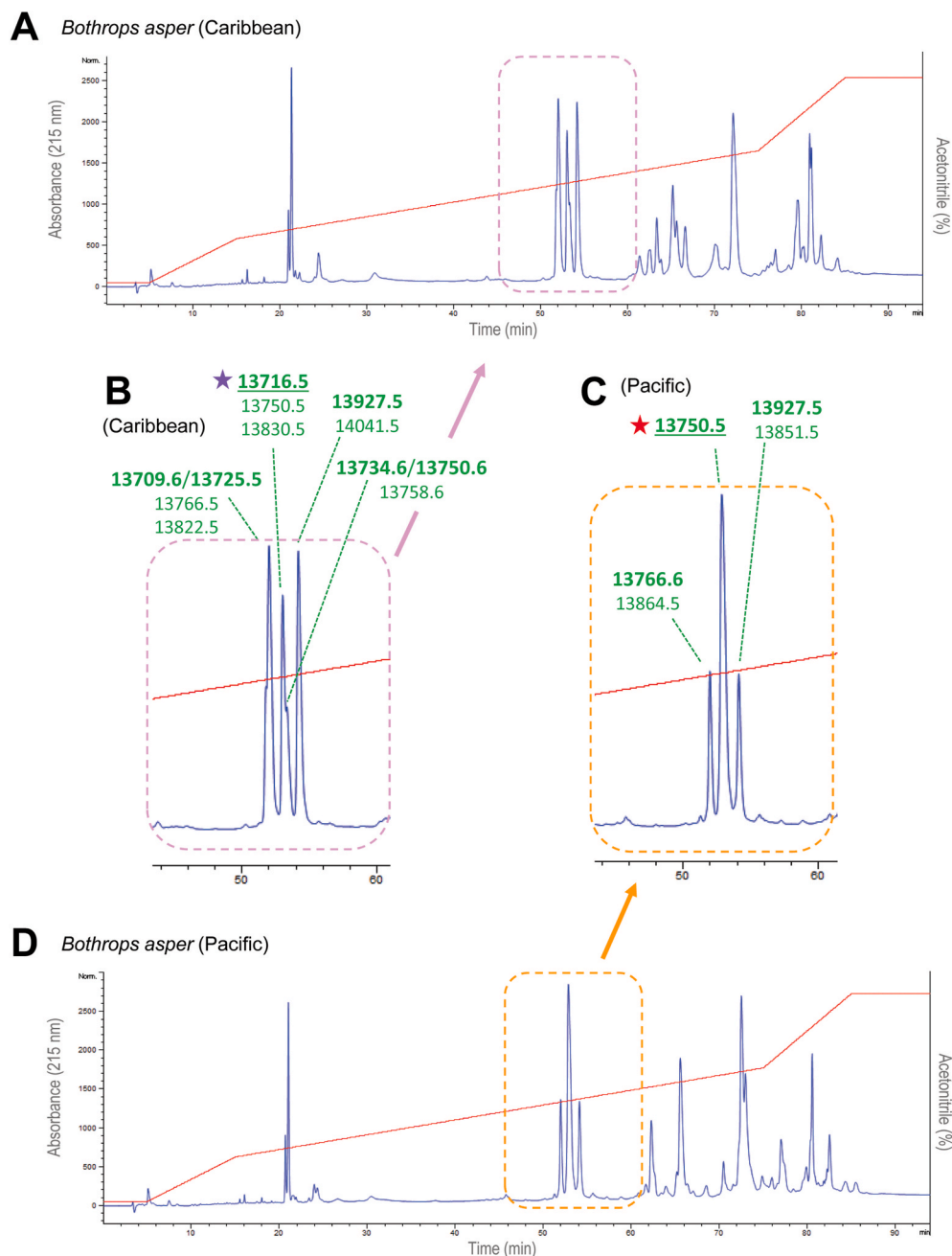


Fig. 3. Analytical C_{18} RP-HPLC of the pooled venoms of *Bothrops asper* from specimens of the Caribbean (A) and Pacific (D) regions of Costa Rica. The acetonitrile gradient applied for elution is shown as a red line. Dashed boxes in the central region of these chromatograms enclose the prominent protein peaks corresponding to myotoxin isoforms present in both venoms, and are zoomed in (B) for the Caribbean venom, and in (C) for the Pacific venom. Intact masses determined in each of the peaks are annotated, with the predominant mass in each case indicated by green boldface. Underlined values marked with a star correspond to the Leu114 variant of myotoxin-II of Caribbean venom in (B), and to the Phe114 variant myotoxin-II of the Pacific venom in (C). The latter protein showed higher homogeneity and yield in the combined RP-HPLC and MS analyses. For interpretation of colors in this figure the reader is referred to the online version of the article.

methods of purification, would be highly recommended for a better clarity on the identity of toxin isoforms studied. Since snake venom PLA₂s and PLA₂-like myotoxins do not show post-translational modifications, their accurate intact mass values should match their amino acid sequence as reliable markers of identity (Calvete et al., 2021).

4.3. The Leu/Phe microheterogeneity of *B. asper* myotoxin-II lies within its toxic site

Since the Leu/Phe microheterogeneity here addressed lies within the bioactive C-terminal region of *B. asper* myotoxin-II (Lomonte et al., 1994), knowing its difference in isoforms isolated from BaP or BaC venom sources is relevant to structure-function studies. Crystallographic analyses on Lys49 myotoxins have shown significant allosteric changes affecting the orientation of amino acid residues of the C-terminal region. In ACL myotoxin, a Lys49 PLA₂-like protein isolated from *Agkistrodon*

contortrix laticinctus venom (Johnson and Ownby, 1993), an allosteric change in the C-terminal region has been proposed to be induced upon binding of a fatty acid at the “(in)active site”, by which the two Phe residues within segment “115–129” reorient to become exposed toward the solvent and form a protruding hydrophobic knuckle (Ambrosio et al., 2005). Similarly, ligand-induced allosteric reorientations of C-terminal amino acids have been demonstrated in Lys49 myotoxins of *Bothrops* venoms from Brazil (Fernandes et al., 2014; Gomes et al., 2020), and Phe124 (here found in *B. asper* myotoxin-II from BaP venom) has been identified as a relevant component of the hydrophobic “membrane disrupting site” of *B. moojeni* myotoxin I (Salvador et al., 2018). Moreover, recent work by Almeida et al. (2022) using synthetic “115–129” C-terminal peptides derived from two Lys49 proteins of *Agkistrodon piscivorus piscivorus* and *A. contortrix laticinctus* (p-App and p-Acl, respectively) which differ only in the Leu/Phe position here analyzed, showed that this substitution can influence their bactericidal and

Accession Species Type name	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125
consensus numbering of region "115–129" (Renetseder et al., 1985)	115	116	117	118	119	120	121	122	123	124	125	128	129							
sp P24605.3 <i>Bothrops asper</i> K49 myotoxin-II (Caribbean)	K	K	Y	R	Y	-	Y	L	K	P	L	C	K	K	-	A	D	A	-	C
sp ----- <i>Bothrops asper</i> K49 myotoxin-II (Pacific)	K	K	Y	R	Y	-	Y	L	K	P	F	C	K	K	-	A	D	A	-	C
sp Q9PVE3.1 <i>Bothrops asper</i> K49 M1-3-3	K	K	Y	K	N	N	Y	L	K	P	F	C	K	K	-	A	D	P	-	C
sp P0C616.1 <i>Bothrops asper</i> K49 myotoxin-IVa	K	K	Y	K	I	-	Y	P	K	F	F	C	K	K	-	A	E	P	-	C
emb CAA55334.2 <i>Bothrops jararacussu</i> K49 bothropstoxin-Ia	K	K	Y	R	Y	-	H	L	K	P	F	C	K	K	-	A	D	A	-	C
sp Q90249.3 <i>Bothrops jararacussu</i> K49 bothropstoxin-I	K	K	Y	R	Y	-	H	L	K	P	F	C	K	K	-	A	D	P	-	C
sp P58399.2 <i>Bothrops pirajai</i> K49 piratoxin-I	K	L	Y	R	Y	-	H	L	K	P	F	C	K	K	-	A	D	D	-	C
sp P82287.1 <i>Bothrops pirajai</i> K49 piratoxin-II	K	K	Y	R	Y	-	H	L	K	P	F	C	K	K	-	A	D	D	-	C
sp Q9IAT9.3 <i>Bothrops pauloensis</i> K49 BnSP-7	K	K	Y	R	Y	-	H	L	K	P	F	C	K	K	-	A	D	P	-	C
sp P0DTS8.1 <i>Bothrops brazili</i> K49 BnSP-7	K	K	Y	R	Y	-	H	L	K	P	F	C	K	K	-	A	D	A	-	C
sp J6L8L6.2 <i>Bothrops brazili</i> K49 MTX-II	K	K	Y	R	Y	-	H	L	K	P	F	C	K	K	-	A	D	P	-	C
sp P86453.1 <i>Bothrops alternatus</i> K49 BaTx	K	K	Y	R	Y	-	Y	L	K	P	L	C	K	K	-	A	D	A	-	C
sp P86975.1 <i>Bothrops leucurus</i> K49 BIK	K	K	Y	R	Y	-	H	L	K	P	F	C	K	K	-	A	D	P	-	C
sp P82114.1 <i>Bothrops moojeni</i> K49 MJTX-I	K	K	R	D	V	-	Y	L	K	P	F	C	D	K	-	G	R	D	-	C
sp Q9I834.2 <i>Bothrops moojeni</i> K49 MJTX-II	K	K	Y	R	Y	N	Y	L	K	P	F	C	K	K	-	A	D	P	-	C
sp AA01S5XW05.1 <i>Bothrops moojeni</i> K49 BomoTx	K	K	Y	K	N	N	Y	L	K	P	F	C	K	K	-	A	D	P	-	C
sp Q6JK69.1 <i>Bothrops atrox</i> K49 myotoxin-I	K	K	Y	K	N	N	Y	L	K	P	F	C	K	K	-	A	D	A	-	C
gb AFJ79209.1 <i>Bothrops diporus</i> K49 Myo-II	K	K	Y	R	Y	N	Y	L	K	P	F	C	K	K	-	A	D	P	-	C
gb QNM80738.1 <i>Bothrops pictus</i> K49	K	K	Y	K	I	-	Y	L	K	P	L	C	K	K	-	A	E	P	-	C
sp P0DUN7.1 <i>Lachesis muta muta</i> K49 LmutTX	K	K	Y	-	-	N	Y	L	K	P	F	C	K	K	-	A	D	P	-	C
gb AGC67020.1 <i>Bothrocophias andianus</i> K49 myotoxin	K	K	Y	R	Y	-	F	L	K	P	L	C	K	K	-	A	D	A	-	C
sp Q8UVU7.1 <i>Cerrophidion godmani</i> K49 Pgo-K49	K	K	Y	K	I	-	H	M	K	F	F	C	K	K	-	P	D	A	-	C
sp P81165.1 <i>Cerrophidion godmani</i> K49 GodMT-II	K	N	Y	K	I	-	Y	P	K	P	L	C	K	K	-	A	D	A	-	C
gb AAW92120.1 <i>Cerrophidion godmani</i> K49 R6K49	N	K	Y	K	R	-	Y	M	K	V	L	C	K	K	-	P	D	A	-	C
sp P82950.1 <i>Atropoides nummifer</i> K49 myotoxin-II	K	N	Y	K	I	-	Y	P	K	P	L	C	K	K	-	A	D	D	-	C
sp P04361.1 <i>Agkistrodon piscivorus piscivorus</i> K49	K	K	Y	K	A	-	Y	F	K	L	K	C	K	K	-	P	D	T	-	C
sp P49121.1 <i>Agkistrodon contortrix latincinctus</i> K49 ACL myotoxin	K	K	Y	K	A	-	Y	F	K	L	K	C	K	K	-	P	E	T	-	C
sp C0HKC2.1 <i>Agkistrodon piscivorus leucostoma</i> K49 ApiP2	K	K	Y	K	A	-	Y	F	K	L	K	C	K	K	-	P	D	T	-	C
sp C0HKC1.1 <i>Agkistrodon piscivorus conanti</i> K49 ApcP2	K	K	Y	K	A	-	Y	F	K	L	K	C	K	K	-	P	D	T	-	C
sp P80963.2 <i>Bothriechis schlegelii</i> K49 Bsc-K49	K	N	Y	K	I	-	T	M	K	F	F	C	K	K	-	P	D	A	-	C
sp P0DJJ8.1 <i>Protobothrops flavoviridis</i> K49 BP-I	K	K	Y	T	I	-	Y	P	K	P	F	C	K	K	-	A	D	T	-	C
gb AUS82463.1 <i>Crotalus mitchellii</i> K49 Cmitc-PLA2-1	K	K	Y	R	V	-	Y	P	K	F	L	C	K	K	-	P	D	T	-	C
sp P0DUP2.1 <i>Crotalus oreganus abyssus</i> K49 CoaTx-II	K	K	Y	R	I	-	Y	P	K	F	L	C	K	K	-	P	D	T	-	C
gb ANN23923.1 <i>Crotalus molossus nigrescens</i> K49 PLA2-2	K	K	Y	K	I	-	Y	P	K	F	L	C	K	K	-	P	D	T	-	C
sp Q8UVZ7.1 <i>Crotalus atrox</i> K49 Cax-K49	K	K	Y	K	I	-	Y	P	K	F	L	C	K	K	-	P	D	T	-	C
sp Q3HLQ4.1 <i>Protobothrops mucrosquamatus</i> N49 TM-N49	K	R	N	I	F	-	Y	P	K	S	S	C	T	E	P	T	D	T	-	C
sp P22640.1 <i>Protobothrops mucrosquamatus</i> K49 TMV-K49	K	K	H	R	V	-	T	V	K	F	L	C	K	A	-	P	E	S	-	C
sp Q2PWA3.1 <i>Protobothrops mucrosquamatus</i> R49 promutoxin	K	K	H	R	V	-	T	M	K	F	L	C	K	A	-	P	E	S	-	C
sp P84776.1 <i>Protobothrops mangshanensis</i> R49 zhaoemiatoxin	K	N	Y	R	F	-	T	M	K	F	L	C	D	K	-	P	E	K	-	C
gb AHJ09548.1 <i>Protobothrops mangshanensis</i> R49	K	N	Y	R	F	-	T	M	K	F	L	C	D	K	-	P	E	Q	-	C
sp Q2PG81.2 <i>Protobothrops elegans</i> R49 PeBP(R)-III	K	N	H	R	V	-	T	V	K	F	L	C	K	A	-	P	E	S	-	C
sp Q6H3D3.1 <i>Trimeresurus stejnegeri</i> K49 Ts-K49c	K	K	K	K	I	-	N	L	K	L	F	C	K	K	T	S	E	Q	-	C
sp Q6H3D1.1 <i>Trimeresurus stejnegeri</i> K49 CTs-K49b	K	K	K	K	I	-	T	L	K	L	S	C	K	K	T	S	E	Q	-	C
gb AHJ09555.1 <i>Trimeresurus gumprechtii</i> K49	K	K	Y	K	L	-	N	L	K	V	F	C	K	K	-	A	D	P	-	C
sp Q2YHJ9.1 <i>Trimeresurus puniceus</i> K49 Tpu-K49a	K	K	E	R	I	-	N	T	K	I	F	C	K	K	T	P	E	P	-	C
gb AHJ09541.1 <i>Trimeresurus sabahi</i> K49	K	K	K	K	I	-	N	L	K	L	F	C	K	K	T	S	E	Q	-	C
gb AHJ09542.1 <i>Trimeresurus sabahi</i> K49	K	K	K	K	I	-	N	L	K	L	F	C	K	K	T	S	G	Q	-	C
sp Q2YHJ4.1 <i>Trimeresurus borneensis</i> K49 Tbo-K49	K	K	E	R	I	-	N	T	K	I	F	C	K	K	T	S	E	P	-	C
sp P70089.1 <i>Trimeresurus gramineus</i> K49 isozyme VII	K	K	K	K	I	-	N	L	K	L	F	C	K	K	T	S	E	K	-	C
gb AHJ09517.1 <i>Trimeresurus albolabris</i> K49	K	K	K	R	I	-	K	P	K	F	F	C	K	K	T	S	E	P	-	C
sp A8E2V9.1 <i>Trimeresurus gracilis</i> K49 Tgc-K49	K	K	Y	K	I	-	Y	L	K	F	L	C	K	K	-	P	E	P	-	C
sp O57385.1 <i>Deinagkistrodon acutus</i> K49 acutohaemolysin	K	S	F	R	Y	-	H	L	K	P	S	C	K	K	T	S	E	Q	-	C
gb AAL36975.1 <i>Deinagkistrodon acutus</i> K49	K	S	F	R	Y	-	H	L	K	P	L	C	K	K	T	S	E	Q	-	C
sp Q92152.1 <i>Ovophis okinavensis</i> K49 PLA2-03	K	K	Y	K	I	-	F	P	K	F	L	C	K	K	-	P	E	P	-	C
gb AUC64914.1 <i>Glyodymus strauchi</i> K49	E	K	Y	K	M	-	Y	L	K	F	L	C	K	K	-	P	E	K	-	C
gb AHJ09558.1 <i>Glyodymus breviceaudus</i> N49	K	R	Y	M	T	-	Y	P	N	I	L	C	S	S	K	S	E	K	-	C
sp O42188.1 <i>Glyodymus halyi</i> N49	K	R	Y	M	T	-	Y	P	N	I	L	C	S	S	K	S	E	K	-	C
sp P0DKU1.1 <i>Glyodymus ussuriensis</i> Q49 Gln49-PLA2	K	I	Y	M	A	-	Y	P	D	I	F	C	S	S	K	S	E	K	-	C
gb AJA90801.1 <i>Azemiops feae</i> N49 Af-N49b	L	R	Y	K	F	-	Y	P	R	F	L	C	K	K	E	S	R	K	-	C
sp P48650.1 <i>Echis carinatus</i> S49 ecarpholin S	K	K	Y	T	Y	-	Y	P	N	F	W	C	K	G	D	I	E	K	-	C
gb AJA90796.1 <i>Echis carinatus</i> S49 Ec-S49	K	K	Y	R	I	-	Y	P	N	F	L	C	R	G	D	P	D	K	-	C
sp P0DMT3.1 <i>Echis coloratus</i> S49	K	K	Y	K	I	-	Y	P	N	I	L	C	R	G	E	P	D	K	-	C
sp P0DMT1.1 <i>Echis pyramidium leakeyi</i> S49	K	K	Y	R	I	-	Y	P	N	F	L	C	R	G	D	P	D	K	-	C
sp B5U6Y4.1 <i>Echis ocellatus</i> S49 Eoc15	K	K	Y	T	Y	-	Y	P	N	F	L	C	K	G	E	P	E	K	-	C
sp P17935.1 <i>Vipera ammodytes ammodytes</i> S49 ammodytin L	K	K	Y	K	V	-	Y	L	R	F	K	C	K	G	V	S	E	K	-	C
emb CAE47262.1 <i>Vipera ammodytes ammodytes</i> S49 ammodytin L1	K	K	Y	K	V	-	H	L	R	F	K	C	K	G	V	S	E	K	-	C
emb CAE47275.1 <i>Vipera ammodytes montandoni</i> S49 ammodytin L3	K	K	Y	K	V	-	Y	L	R	F	K	C	K	G	V	S	E	R	-	C
emb CAE47273.1 <i>Vipera ammodytes montandoni</i> S49 ammodytin L3	K	K	Y	K	V	-	Y	L	G	F	K	C	K	G	V	S	E	K	-	C
emb CAE47257.1 <i>Vipera ammodytes ruffoi</i> S49 ammodytin L4	K	K	Y	K	V	-	Y	L	R	F	K	C	K	G	V	S	E	K	-	C
emb CAE47253.1 <i>Vipera berus berus</i> S49 ammodytin L2	K	K	Y	K	V	-	Y	P	R	F	K	C	K	G	V	S	E	K	-	C
sp F8QN50.1 <i>Vipera renardi</i> S49 Vur-S49	K	K	Y	K	V	-	Y	L	R	F	K	C	K	G	V	P	E	K	-	C

Fig. 4. Aligned amino acid sequences of the C-terminal region of 70 “non-Asp49” phospholipase A₂-like proteins from viperid snake species. Position numbers with black background at the top refer to the numbering of the alignment, whereas their equivalent numbers in the consensus system of Renetseder et al. (1983) are shown with a light blue background (region “115–129”) just below. The presence of leucine (L) or phenylalanine (F) at consensus position 124 in the different proteins is highlighted with yellow or pink backgrounds, respectively. Conserved cysteine (C) residues are shown with a gray background. For interpretation of colors in this figure the reader is referred to the online version of the article.

cytotoxic activities, with the Phe-containing peptide being more active in some *in vitro* assays. Both p-App and p-Acl peptides were previously observed to induce a myotoxic effect *in vivo* (Lomonte et al., 2003a, 2003b). Overall, the above examples highlight the relevance of having accurately defined structural information on myotoxins' bioactive region microheterogeneity when investigating their effects and modes of action, as recently discussed by Borges et al. (2022).

4.4. Variability in Leu/Phe within the C-terminal region of PLA₂-like toxins

A sequence comparison based on more limited data available two

decades ago had already noted that the C-terminal region of Lys49 proteins displays high variability (Lomonte et al., 2003a, 2003b). More recent comparative analyses (Peggion and Tonello, 2021) and the present work, based on 70 non-Asp49 PLA₂-like snake venom proteins, concur with this previous finding, and expand the view on the primary structure diversity of region “115–129” in a broader variety of true viper and pitviper taxons (Fig. 4). Either Leu or Phe occupy the position 124 (consensus) preceding the conserved cysteine (number 116 in the alignment of Fig. 4, or 125 in the consensus numbering) in the majority of PLA₂-like proteins, with roughly equal frequencies (Fig. 5), while fewer members of this group present lysine at this position. Knowing that myotoxicity is reported for a number of *Bothrops* proteins having

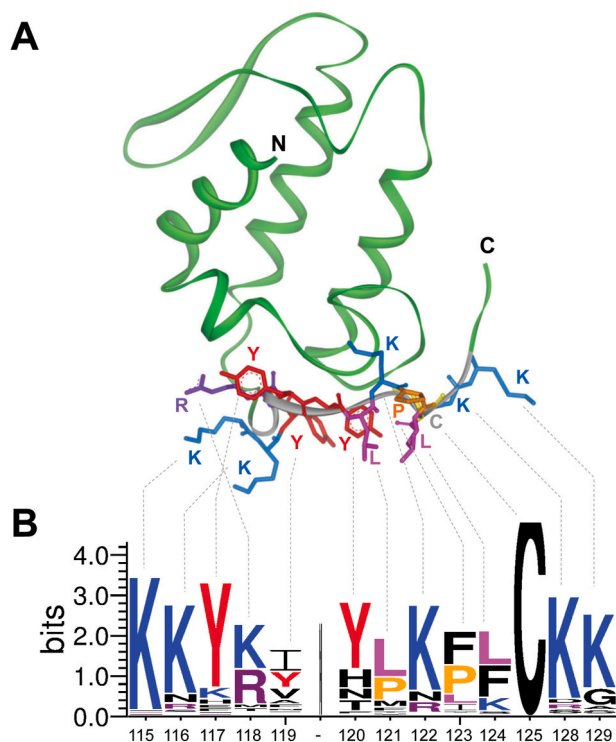


Fig. 5. (A) Ribbon representation of the three-dimensional structure of a *Bothrops asper* myotoxin-II monomer (PDB code 1CLP; Arni et al., 1985) showing the side chains of amino acids of its C-terminal region “115–129”. N- and C-termini are labeled in black. (B) The variability of the C-terminal region among all PLA₂-like proteins compared in Fig. 4 is represented in a WebLogo plot to visualize the frequency of amino acid occurrence for each position. The linear sequence of the plot is linked to the protein structure by dashed gray lines for each amino acid position. For interpretation of colors in this figure the reader is referred to the online version of the article.

either Phe or Leu at position 124 (Fig. 4), it becomes evident that the toxic mechanism is permissive to this variation. Moreover, ACL myotoxin presents Lys124, further indicating that any of these three amino acids (Leu, Phe, or Lys) at position 124 is compatible with the expression of the myotoxic mechanism. However, the functional consequences of the Leu/Phe microheterogeneity in myotoxin-II, in terms of myotoxic potency for example, remain to be experimentally addressed in future work, either by careful purification of the two natural variants or by performing site-directed mutagenesis on recombinantly produced proteins.

The overall variability noted in region “115–129” would appear at first sight counterintuitive with the concept of an expected conservation for a functional toxin site. However, by inspecting the frequencies of amino acid residues within this region in members of the PLA₂-like subfamily (Fig. 5) the overall sequence organization supports the idea of a site where the combination of mostly conserved cationic residues (lysines) at both ends of this stretch, with a more variable blend of hydrophobic and basic residues in the middle, leads to a membrane-active functional site that is not strictly dependent on a fixed sequence. This kind of organization bears resemblance to that of a variety of short antimicrobial peptides which rely on diverse combinations of cationic and hydrophobic amino acids to affect the viability of microorganisms (Jenssen et al., 2006; Fjell et al., 2012), as first noted by Kini and Evans (1989). Indeed, Lys49 myotoxins or their C-terminal-derived peptides have been reported to display some degree of bactericidal (Páramo et al., 1998; Santos-Filho et al., 2021), parasitocidal (Costa et al., 2008; Grabner et al., 2017), fungicidal (Murillo et al., 2007) and virucidal (Cecilio et al., 2013; Freire et al., 2021) activities.

4.5. C-terminal region of PLA₂-like toxins: sequence plasticity for a functional toxic site

Sequence comparisons based on a growing number of PLA₂-like toxins, together with the growing three-dimensional structural information (Fernandes et al., 2014), strengthen the notion that the disruptive interaction of Lys49 myotoxins towards membranes cannot be attributed to a fixed amino acid sequence motif, but rather to a flexible spatial array of physicochemical properties provided by variable combinations of cationic and hydrophobic amino acids within a functional site that largely involves the C-terminal region (Lomonte et al., 2003a, 2003b; Lomonte and Rangel, 2012; Fernandes et al., 2014). This plasticity appears to allow some degree of tolerance for variations in the particular amino acids that constitute the toxic site, as long as certain physicochemical and spatial requirements are met. In support of this view, it is noteworthy that mutagenesis experiments with bothropstoxin-I, a Lys49 myotoxin from *B. jararacussu* venom, found that none of the single substitutions investigated within the “115–129” region caused a radical drop in myotoxicity: single mutations at positions 117, 118, and 122, led to significant but slight decreases, being the Lys122Ala mutation the one causing the largest decrease, not exceeding a 30% reduction compared to the native toxin (Chioato et al., 2002). A general consequence of this plasticity of the toxic site of PLA₂-like myotoxins is that structural particularities among different proteins of this group will preclude making straightforward mechanistic extrapolations from one to all others. More structural and biochemical work, especially complemented by wet-lab approaches based on site-directed mutagenesis, among others, will be required to refine and complete the current picture on the key determinants of myotoxicity in PLA₂-like proteins.

Ethical statement

The sponsor had no role in study design, in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the article for publication.

Credit author statement

Bruno Lomonte: Conceptualization, Methodology, Formal analysis, writing and editing. Julián Fernández: Formal analysis, writing and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- Abubakar, S.B., Habib, A.G., Mathew, J., 2010. Amputation and disability following snakebite in Nigeria. *Trop. Doct.* 40, 114–116.
- Alape-Girón, A., Sanz, L., Escolano, J., Flores-Díaz, M., Madrigal, M., Sasa, M., Calvete, J. J., 2008. Snake venomomics of the lancehead pitviper *Bothrops asper*: geographic, individual, and ontogenetic variations. *J. Proteome Res.* 7, 3556–3571.
- Almeida, J.R., Mendes, B., Lancellotti, M.C., Franchi, G., Passos, O., Ramos, M.J., Fernandes, P.A., Alves, C., Vale, N., Gomes, P., da Silva, S., 2022. Lessons from a single amino acid substitution: anticancer and antibacterial properties of two phospholipase A₂-derived peptides. *Curr. Issues Mol. Biol.* 44, 46–62.

- Almeida, J.R., Mendes, B., Lancellotti, M., Marangoni, S., Vale, N., Passos, Ó., Ramos, M. J., Fernandes, P.A., Gomes, P., Da Silva, S.L., 2018. A novel synthetic peptide inspired on Lys49 phospholipase A₂ from *Crotalus oreganus abyssus* snake venom active against multidrug-resistant clinical isolates. *Eur. J. Med. Chem.* 149, 248–256.
- Ambrosio, A.L.B., Nonato, M.C., Selistre de Araujo, H.S., Arni, R.K., Ward, R.J., Ownby, C.L., de Souza, D.H.F., Garrat, R.C., 2005. A molecular mechanism for Lys49-phospholipase A₂ activity based on ligand induced conformational change. *J. Biol. Chem.* 280, 7326–7335.
- Arni, R.K., Ward, R.J., Gutiérrez, J.M., Tulinsky, A., 1995. Structure of a calcium-independent phospholipase-like myotoxic protein from *Bothrops asper* venom. *Acta Crystallogr. D* 51, 311–317.
- Borges, R.J., Lemke, N., Fontes, M.R.M., 2017. PLA₂-like proteins myotoxic mechanism: a dynamic model description. *Sci. Rep.* 7, 15514.
- Borges, R.J., Salvador, G.H.M., Campanelli, H.B., Pimenta, D.C., de Oliveira Neto, M., Usón, I., Fontes, M.R.M., 2021. BthTX-II from *Bothrops jararacussu* venom has variants with different oligomeric assemblies: an example of snake venom phospholipases A₂ versatility. *Int. J. Biol. Macromol.* 191, 255–266.
- Borges, R.J., Salvador, G.H.M., Pimenta, D.C., Dos Santos, L.D., Fontes, M.R.M., Usón, I., 2022. Sequence slider: integration of structural and genetic data to characterize isoforms from natural sources. *Nucleic Acids Res.* <https://doi.org/10.1093/nar/gkac029> (online ahead of print).
- Bustillo, S., Fernández, J., Chaves, S., Angulo, Y., Leiva, L.C., Lomonte, B., 2019. Isolation of two basic phospholipases A₂ from *Bothrops diporus* snake venom: comparative characterization and synergism between Asp49 and Lys49 variants. *Toxicon* 168, 113–121.
- Calvete, J.J., Sanz, L., Mora-Obando, D., Lomonte, B., Tanaka-Azevedo, A.M., de Moraes-Zani, K., Sant'Anna, S.S., Caldeira, C.A.S., 2021. What's in a mass? *Biochem. Soc. Trans.* 49, 1027–1037.
- Cecilio, A.B., Caldas, S., Oliveira, R.A., Santos, A.S., Richardson, M., Naumann, G.B., Schneider, F.S., Alvarenga, V.G., Estevão-Costa, M.I., Fuly, A.L., Eble, J.A., Sanchez, E.F., 2013. Molecular characterization of Lys49 and Asp49 phospholipases A₂ from snake venom and their antiviral activities against Dengue virus. *Toxins* 5, 1780–1798.
- Chijiwa, T., Yamaguchi, Y., Ogawa, T., Deshimaru, M., Nobuhisa, I., Nakashima, K., Oda-Ueda, N., Fukumaki, Y., Hattori, S., Ohno, M., 2003. Interisland evolution of *Trimeresurus flavoviridis* venom phospholipase A₂ isozymes. *J. Mol. Evol.* 56, 286–293.
- Chioato, L., de Oliveira, A.H.C., Ruller, R., Sá, J.M., Ward, R.J., 2002. Distinct sites for myotoxic and membrane-damaging activities in the C-terminal region of a Lys49-phospholipase A₂. *Biochem. J.* 366, 971–976.
- Chioato, L., Aragão, E.A., Ferreira, T.L., de Medeiros, A.I., Faccioli, L.H., Ward, R.J., 2007. Mapping of the structural determinants of artificial and biological membrane damaging activities of a Lys49 phospholipase A₂ by scanning alanine mutagenesis. *Biochim. Biophys. Acta* 1768, 1247–1257.
- Cintra-Francischini, M., Pizzo, P., Rodrigues-Simioni, L., Ponce-Soto, L., Rossetto, O., Lomonte, B., Gutiérrez, J.M., Pozzan, T., Montecucco, C., 2009. Calcium imaging of muscle cells treated with snake myotoxins reveals toxin synergism and presence of receptors. *Cell. Mol. Life Sci.* 66, 1718–1728.
- Cintra-Francischini, M., Pizzo, P., Angulo, Y., Gutiérrez, J.M., Montecucco, C., Lomonte, B., 2010. The C-terminal region of a Lys49 myotoxin mediates Ca²⁺ influx in C2C12 myotubes. *Toxicon* 55, 590–596.
- Costa, T.R., Menaldo, D.L., Oliveira, C.Z., Santos-Filho, N.A., Teixeira, S.S., Nomizo, A., Fuly, A.L., Monteiro, M.C., de Souza, B.M., Palma, M.S., Stábeli, R.G., Sampaio, S.V., Soares, A.M., 2008. Myotoxic phospholipases A₂ isolated from *Bothrops brasili* snake venom and synthetic peptides derived from their C-terminal region: cytotoxic effect on microorganism and tumor cells. *Peptides* 29, 1645–1656.
- de Oliveira, A.H.C., Aragão, E.A., Sá, J.M., Chioato, L., Bugs-Bortoleto, R., Ruller, R., Ferreira, T.L., Ward, R.J., 2009. Calcium-independent membrane damage by venom phospholipases A₂. *Protein Pept. Lett.* 16, 877–886.
- dos Santos, J.I., Soares, A.M., Fontes, M.R., 2009. Comparative structural studies on Lys49-phospholipases A₂ from *Bothrops genus* reveal their myotoxic site. *J. Struct. Biol.* 167, 106–116.
- Fernandes, C.A., Borges, R.J., Lomonte, B., Fontes, M.R., 2014. A structure-based proposal for a comprehensive myotoxic mechanism of phospholipase A₂-like proteins from viperid snake venoms. *Biochim. Biophys. Acta* 1844, 2265–2276.
- Fernández, J., Caccin, P., Koster, G., Lomonte, B., Gutiérrez, J.M., Montecucco, C., Postle, A.D., 2013. Muscle phospholipid hydrolysis by *Bothrops asper* Asp49 and Lys49 phospholipase A₂ myotoxins - distinct mechanisms of action. *FEBS J.* 280, 3878–3886.
- Fjell, C.D., Hiss, J.A., Hancock, R.E., Schneider, G., 2012. Designing antimicrobial peptides: form follows function. *Nat. Rev. Drug Discov.* 11, 37–51.
- Francis, B., Gutiérrez, J.M., Lomonte, B., Kaiser II, 1991. Myotoxin II from *Bothrops asper* (Terciopelo) venom is a lysine-49 phospholipase A₂. *Arch. Biochem. Biophys.* 284, 352–359.
- Freire, M.C.L.C., Noske, G.D., Bitencourt, N.V., Sanches, P.R.S., Santos-Filho, N.A., Gawriljuk, V.O., de Souza, E.P., Nogueira, V.H.R., de Godoy, M.O., Nakamura, A.M., Fernandes, R.S., Godoy, A.S., Juliano, M.A., Peres, B.M., Barbosa, C.G., Moraes, C.B., Freitas-Junior, L.H.G., Cilli, E.M., Guido, R.V.C., Oliva, G., 2021. Non-Toxic dimeric peptides derived from the bothropstoxin-I are potent SARS-CoV-2 and papain-like protease inhibitors. *Molecules* 26, 4896.
- Gebri, L.C., Marcussi, S., Menaldo, D.L., de Menezes, C.S., Nomizo, A., Hamaguchi, A., Silveira-Lacerda, E.P., Homsi-Brandeburgo, M.I., Sampaio, S.V., Soares, A.M., Rodrigues, V.M., 2009. Antitumor effects of snake venom chemically modified Lys49 phospholipase A₂-like BthTX-I and a synthetic peptide derived from its C-terminal region. *Biologicals* 37, 222–229.
- Gomes, A.A.S., Cardoso, F.F., Souza, M.F., Oliveira, C.L.P., Perahia, D., Magro, A.J., Fontes, M.R.M., 2020. The allosteric activation mechanism of a phospholipase A₂-like toxin from *Bothrops jararacussu* venom: a dynamic description. *Sci. Rep.* 10, 16252.
- Grabner, A.N., Alfonso, J., Kayano, A.M., Moreira-Dill, L.S., Dos Santos, A.P.A., Caldeira, C.A.S., Sobrinho, J.C., Gómez, A., Grabner, F.P., Cardoso, F.F., Zuliani, J.P., Fontes, M.R.M., Pimenta, D.C., Gómez, C.V., Teles, C.B.G., Soares, A.M., Calderon, L. A., 2017. BmajPLA₂-II, a basic Lys49-phospholipase A₂ homologue from *Bothrops marajoensis* snake venom with parasitocidal potential. *Int. J. Biol. Macromol.* 102, 571–581.
- Harris, J.B., Cullen, M.J., 1990. Muscle necrosis caused by snake venoms and toxins. *Electron. Microsc. Rev.* 3, 183–211.
- Ikeda, N., Chijiwa, T., Matsubara, K., Oda-Ueda, N., Hattori, S., Matsuda, Y., Ohno, M., 2010. Unique structural characteristics and evolution of a cluster of venom phospholipase A₂ isozyme genes of *Protobothrops flavoviridis* snake. *Gene* 461, 15–25.
- Jensen, H., Hamill, P., Hancock, R.E., 2006. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* 19, 491–511.
- Johnson, E.K., Ownby, C.L., 1993. Isolation of a myotoxin from the venom of *Agkistrodon contortrix laticinctus* (broad-banded copperhead) and pathogenesis of myonecrosis induced by it in mice. *Toxicon* 31, 243–255.
- Kini, R.M., Evans, H.J., 1989. A common cytolytic region in myotoxins, hemolysins, cardiotoxins and antibacterial peptides. *Int. J. Pept. Protein Res.* 34, 277–286.
- Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. Mega X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549.
- Lomonte, B., Carmona, E., 1992. Individual expression patterns of myotoxin isoforms in the venom of the snake *Bothrops asper*. *Comp. Biochem. Physiol.* 102B, 325–329.
- Lomonte, B., Gutiérrez, J.M., 1989. A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon* 27, 725–733.
- Lomonte, B., Gutiérrez, J.M., 2011. Phospholipases A₂ from Viperidae snake venoms: how do they induce skeletal muscle damage? *Acta Chim. Slov.* 58, 647–659.
- Lomonte, B., Krizaj, I., 2021. Snake venom phospholipase A₂ toxins. In: Mackessy, S. (Ed.), *Handbook of Venoms and Toxins of Reptiles*. CRC Press, Boca Raton, pp. 389–411.
- Lomonte, B., Rangel, J., 2012. Snake venom Lys49 myotoxins: from phospholipases A₂ to non-enzymatic membrane disruptors. *Toxicon* 60, 520–530.
- Lomonte, B., Calvete, J.J., 2017. Strategies in 'snake venomomics' aiming at an integrative view of compositional, functional, and immunological characteristics of venoms. *JVATiTD* 23, 26.
- Lomonte, B., Moreno, E., Tarkowski, A., Hanson, L.Å., Maccarana, M., 1994. Neutralizing interaction between heparins and myotoxin II, a Lys-49 phospholipase A₂ from *Bothrops asper* snake venom. Identification of a heparin-binding and cytolytic toxin region by the use of synthetic peptides and molecular modeling. *J. Biol. Chem.* 269, 29867–29873.
- Lomonte, B., Angulo, Y., Santamaría, C., 2003a. Comparative study of synthetic peptides corresponding to region 115–129 in Lys49 myotoxic phospholipases A₂ from snake venoms. *Toxicon* 42, 307–312.
- Lomonte, B., Angulo, Y., Calderón, L., 2003b. An overview of Lysine-49 phospholipase A₂ myotoxins from crotalid snake venoms and their structural determinants of myotoxic action. *Toxicon* 42, 885–901.
- Maraganore, J.M., Merutka, G., Cho, W., Welches, W., Kézdy, F.J., Heinrikson, R.L., 1984. A new class of phospholipases A₂ with lysine in place of aspartate 49. *J. Biol. Chem.* 259, 13839–13843.
- Means, R., Cabrera, J., Moreno, X., Amini, R., 2017. Remote South American snakebite with extensive myonecrosis. *Clin. Pract. Cases Emerg. Med.* 1, 47–49.
- Mebs, D., Ownby, C.L., 1990. Myotoxic components of snake venoms: their biochemical and biological activities. *Pharmacol. Ther.* 48, 223–236.
- Montecucco, C., Gutiérrez, J.M., Lomonte, B., 2008. Cellular pathology induced by snake venom phospholipase A₂ myotoxins and neurotoxins: common aspects of their mechanisms of action. *Cell. Mol. Life Sci.* 65, 2897–2912.
- Mora-Obando, D., Fernández, J., Montecucco, C., Gutiérrez, J.M., Lomonte, B., 2014. Synergism between basic Asp49 and Lys49 phospholipase A₂ myotoxins of viperid snake venom in vitro and in vivo. *PLoS One* 9, e109846.
- Murakami, M.T., Arruda, E.Z., Melo, P.A., Martinez, A.B., Lomonte, B., Gutiérrez, J.M., Arni, R.K., 2005. Inhibition of myotoxic activity of *Bothrops asper* myotoxin II by the anti-trypanosomal drug suramin. *J. Mol. Biol.* 350, 416–426.
- Murillo, L.A., Lan, C.Y., Agabian, N.M., Larios, S., Lomonte, B., 2007. Fungicidal activity of a phospholipase A₂-derived synthetic peptide variant upon *Candida albicans*. *Rev. Española Quimioter.* 20, 330–333.
- Nakashima, K.I., Nobuhisa, I., Deshimaru, M., Nakai, M., Ogawa, T., Shimohigashi, Y., Fukumaki, Y., Hattori, M., Sakaki, Y., Hattori, S., Ohno, M., 1995. Accelerated evolution in the protein-coding regions is universal in crotalinae snake venom gland phospholipase A₂ isozyme genes. *Proc. Natl. Acad. Sci. U.S.A.* 92, 5605–5609.
- Núñez, C.E., Angulo, Y., Lomonte, B., 2001. Identification of the myotoxic site of the Lys49 phospholipase A₂ from *Agkistrodon piscivorus piscivorus* snake venom: synthetic C-terminal peptides from Lys49, but not from Asp49 myotoxins, exert membrane-damaging activities. *Toxicon* 39, 1587–1594.
- Ohno, M., Chijiwa, T., Oda-Ueda, N., Ogawa, T., Hattori, S., 2003. Molecular evolution of myotoxic phospholipases A₂ from snake venom. *Toxicon* 42, 841–854.
- Páramo, L., Lomonte, B., Pizarro-Cerdá, J., Bengoechea, J.A., Gorvel, J.P., Moreno, E., 1998. Bactericidal activity of Lys49 and Asp49 myotoxic phospholipases A₂ from *Bothrops asper* snake venom: synthetic Lys49 myotoxin II-(115–129)-peptide identifies its bactericidal region. *Eur. J. Biochem.* 253, 452–461.
- Peggion, C., Tonello, F., 2021. Short linear motifs characterizing snake venom and mammalian phospholipases A₂. *Toxins* 13, 290.

- Renetseder, R., Brunie, S., Dijkstra, B.W., Drenth, J., Sigler, P.B., 1985. A comparison of the crystal structures of phospholipase A₂ from bovine pancreas and *Crotalus atrox* venom. *J. Biol. Chem.* 260, 11627–11634.
- Rufini, S., Cesaroni, P., Desideri, A., Farias, R., Gubensek, F., Gutiérrez, J.M., Luly, P., Massoud, R., Morero, R., Pedersen, J.Z., 1992. Calcium ion independent membrane leakage induced by phospholipase-like myotoxins. *Biochemistry* 31, 12424–12430.
- Salvador, G.H.M., Dreyer, T.R., Gomes, A.A.S., Cavalcante, W.L.G., Dos Santos, J.I., Gandin, C.A., de Oliveira Neto, M., Gallacci, M., Fontes, M.R.M., 2018. Structural and functional characterization of suramin-bound MjTX-I from *Bothrops moojeni* suggests a particular myotoxic mechanism. *Sci. Rep.* 8, 10317.
- Salvador, G.H.M., Borges, R.J., Lomonte, B., Lewin, M.R., Fontes, M.R.M., 2021. The synthetic varespladib molecule is a multi-functional inhibitor for PLA₂ and PLA₂-like ophidic toxins. *Biochim. Biophys. Acta* 1865, 129913.
- Santos-Filho, N.A., de Freitas, L.M., Santos, C.T.D., Piccoli, J.P., Fontana, C.R., Fusco-Almeida, A.M., Cilli, E.M., 2021. Understanding the mechanism of action of peptide (p-BthTX-I)₂ derived from C-terminal region of phospholipase A₂ (PLA₂)-like bothropstoxin-I on Gram-positive and Gram-negative bacteria. *Toxicon* 196, 44–55.
- Soares, A.M., Rodrigues, V.M., Homs-Brandeburgo, M.I., Toyama, M.H., Lombardi, F.R., Arni, R.K., Giglio, J.R., 1998. A rapid procedure for the isolation of the Lys-49 myotoxin II from *Bothrops moojeni* (caissaca) venom: biochemical characterization, crystallization, myotoxic and edematogenic activity. *Toxicon* 36, 503–514.
- Soares, B.S., Rocha, S.L.G., Bastos, V.A., Lima, D.B., Carvalho, P.C., Gozzo, F.C., Demeler, B., Williams, T.L., Arnold, J., Henrickson, A., Jorgensen, T.J.D., Souza, T.A. C.B., Perales, J., Valente, R.H., Lomonte, B., Gomes-Neto, F., Neves-Ferreira, A.G.C., 2022. Molecular architecture of the antiophidic protein DM64 and its binding specificity to myotoxin II from *Bothrops asper* venom. *Front. Mol. Biosci.* 8, 787368.
- Ward, R.J., Chioato, L., de Oliveira, A.H.C., Ruller, R., Sá, J.M., 2002. Active site mutagenesis of a Lys49-phospholipase A₂: biological and membrane-disrupting activities in the absence of catalysis. *Biochem. J.* 362, 89–96.
- Warrell, D.A., 2010. Snakebite. *Lancet* 375, 77–88.