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Electrospray ionization quadrupole time-of-flight and matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometric analyses to solve micro-heterogeneity in post-translationally modified peptides from *Phoneutria nigriventer* (Aranea, Ctenidae) venom

Adriano M. C. Pimenta[1,2]*, Breno Rates[1,2], Carlos Bloch Jr.[3,4], Paulo C. Gomes[5], Marcelo M. Santoro[1,2], Maria Elena de Lima[1,2], Michael Richardson[5] and Marta do N. Cordeiro[5]

[1]Núcleo de Biomoléculas, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627, 31270-901, Belo Horizonte, MG, Brazil
[2]Laboratório de Venenos e Toxinas Animais, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627, 31270-901, Belo Horizonte, MG, Brazil
[3]Laboratório Nacional de Luz Sincrotron, Rua Giuseppe Máximo Scolfaro, Pólo II de Alta Tecnologia de Campinas, Caixa Postal 6192, 13084-971, Campinas, SP, Brazil
[4]Laboratório de Espectrometria de Massa, Embrapa—Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil
[5]Centro de Pesquisa Professor Carlos R. Diniz, Fundação Ezequiel Dias, Rua Conde Pereira Carneiro 80, 30550-010, Belo Horizonte, MG, Brazil

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Previous studies of the fractionated venom of the Brazilian armed spider *Phoneutria nigriventer*, obtained by gel filtration, have demonstrated the presence of a fraction PhM, a pool of small peptides (up to 2000 Da) that provoke contractions in smooth muscle of guinea pig ileum. Initial attempts to sequence these peptides were largely unsuccessful because of the low purification yield and the fact that the majority seemed to be blocked at their N-termini. In the present work, analysis of this venom fraction by mass spectrometry has revealed the existence of a highly complex mixture of peptides with molecular weights corresponding to those observed for the muscle-active peptides previously described (800–1800 Da). These peptides appear to be a family of isoforms with some particular features. The amino acid sequences of 15 isoforms have been determined by tandem mass spectrometry (MS/MS) using both electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q/ToFMS) and matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-ToF/ToFMS). These molecules contain post-translational modifications such as proteolysis and C-terminal amidation, which combine to generate additional isoforms. All the isoforms sequenced in this study possess an N-terminal pyroglutamic acid residue. A search for sequence similarities with other peptides in databanks revealed that these peptides are structurally related to the tachykinins, a family of neuro-hormone peptides. The data obtained in this study will be essential for the subsequent steps of this research, the synthesis of these peptides and pharmacological characterization of their biological activity. Copyright © 2004 John Wiley & Sons, Ltd.

In recent decades, toxinologists have put much effort into determining the structures and functions of the most lethal toxins found in animal venoms. In spite of this, small peptides that are responsible for micro-toxicological effects have been relatively neglected and their biochemical and pharmacological features were, until now, mostly unconsidered. However, the latest developments in micro-scale analytical techniques such as mass spectrometry, and the use of proteomic approaches in toxinology, have changed some of the toxinologists' paradigms. Structure-guided approaches have demonstrated the richness of these venoms, from the structural and pharmacological points of view. The interest in small non-reticulated peptides

**Correspondence to*: A. M. C. Pimenta, Laboratório de Venenos e Toxinas Animais, Depto. de Bioquímica e Imunologia, ICB, UFMG, Av. Antonio Carlos, 6627, 31270-901, Belo Horizonte, Minas Gerais, Brazil.
E-mail: apimenta@icb.ufmg.br
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with vasoactive, hormone-like or antimicrobial effects has increased enormously, mainly due to their potential in biotechnological applications.

The aggressive spiders from the genus *Phoneutria* (Perty, 1833), family Ctenidae, are widely distributed in the warm regions of South America. Several species have been described, including *Phoneutria nigriventer* (Keyserling, 1891), the most common in central and south-eastern regions of Brazil. This spider is frequently involved in human accidents.[1] Previous work with its venom led to the purification and determination of the primary structures of more than 15 toxins, distributed in four main functional groups (PhTx1, PhTx2, PhTx3 and PhTx4). Several of these toxins are highly lethal, to both mammals and arthropods, because of their intense neurotoxic effects, and have molecular masses ranging from 3500–9000 Da.[2–8] In addition to these lethal neurotoxins, small peptides (up to 1700 Da) with smooth-muscle activity were also detected as a non-neurotoxic fraction, named fraction PhM;[3,5] also, it was suggested that these peptides might exist as a pool of highly similar isoforms, a characteristic that had also been observed for the other toxin families of this venom. Attempts to characterize these peptides were largely unsuccessful, both because of their low levels in the whole venom and because their N-termini were somehow blocked which prevented sequencing by Edman degradation.

The main objective of this study was to structurally characterize the smooth-muscle active peptide family present in the previously described fraction PhM from *Phoneutria nigriventer* venom. For this purpose we have made use of the sensitivity, accuracy and resolving power of electrospray ionization quadrupole time-of-flight (ESI-Q/ToF) and matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-ToF/ToF) mass spectrometry (MS) to shed light on this intricate family of non-reticulated peptides.

EXPERIMENTAL

Materials and venom fractionation

Venom collection and storage were performed as described previously.[4–6] Venom fractionation was achieved by loading the soluble fraction onto a semi-preparative reverse-phase high-performance liquid chromatography (RP-HPLC) C4 Vydac column (200 × 20 mm; Technicol Ltd., Stockport, UK). The column was equilibrated with trifluoroacetic acid (TFA) (0.1%), and the protein content was eluted by a linear gradient of 0.1% TFA/acetonitrile (see Fig. 1 for details). The flow rate was 5 mL/min, and elution was monitored by absorbance readings at 214 nm; fractions were collected manually.

Mass spectrometry: instrumentation and analyses

ESI-Q/ToFMS analyses

ESI-Q/ToFMS analyses were performed using a Q-ToF Micro™ instrument (Micromass, Manchester, UK) equipped with an ESI source operated in positive ion mode. The capillary voltage was 3–3.5 kV and sample cone voltages were 30–40 V. Alternatively, a nanospray source was used to increase sensitivity. Mass spectrometer calibrations used sodium iodide with cesium iodide in the *m/z* 100–2000 range. Samples were diluted in 50% acetonitrile/0.1% TFA in Milli-Q® water and introduced using a syringe pump with flow rates of 5–10 μL/min (electrospray) or 0.5–1.0 μL/min (nano-electrospray). Collision-induced dissociation (CID), performed to achieve *de novo* sequencing of peptides, used argon with collision energies in the range 20–50 eV. Data were analyzed by MassLynx® 3.5 software.

MALDI-ToF/ToFMS analyses

MALDI-ToF/ToFMS analyses were performed using an ABI 4700 proteomics analyzer with ToF/ToF optics (Applied Biosystems, USA), as previously described.[9] Briefly, samples diluted in acetic acid (0.1%) were spotted onto a sample plate,

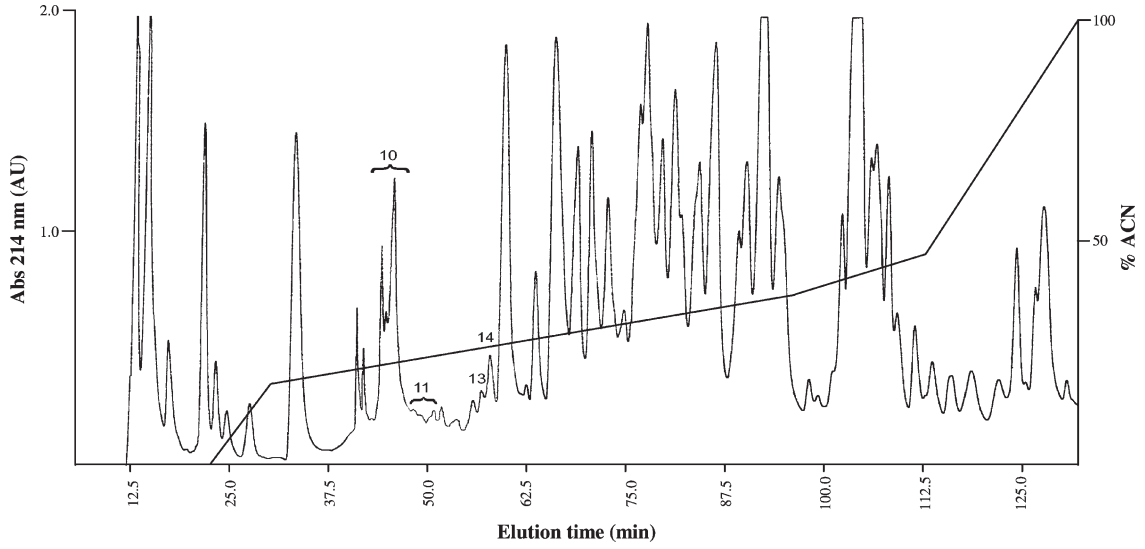

Figure 1. Reverse-phase HPLC chromatogram of the soluble fraction from *Phoneutria nigriventer* venom. Approximately 18.5 mg were loaded on a semi-preparative Vydac C4 column. Absorbance was monitored at 214 nm. The flow rate was 5 mL/min and fractions were collected manually. The peak numbering indicates the fractions that were submitted to mass fingerprinting.

mixed with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid (CHCA), and allowed to dry at room temperature (dried-droplet method). The MS and the MS/MS spectra were acquired in the reflector mode with external calibration, using the calibration mixture Sequazyme standard kit (Applied Biosystems, USA). Peptide *de novo* sequencing was performed by precursor ion fragmentation using N_2 as CID gas with collision cell pressure kept at 2.8×10^{-6} Torr. Post-source decay (PSD) spectra were obtained by increasing the laser energy and turning off the collision gas, as specified by the manufacturer.

The three MS/MS techniques were used to obtain complementary information.

RESULTS AND DISCUSSION

Initial fractionation and partial mass fingerprinting of *P. nigriventer* venom

The soluble fraction of the *P. nigriventer* venom was submitted to RP-HPLC on a C4 column (see LC-UV chromatogram in Fig. 1). The eluted fractions were subjected to MS analyses in order to establish an initial mass fingerprinting used to facilitate this study; Table 1 lists the observed masses present in those fractions in which the peptides with low molecular weights were found (see also Fig. 1). A total of 79 main molecular species were observed in the selected fractions with molecular masses ranging from 301.31–7543.18 Da. Nevertheless, since the aim of this work was to characterize only the peptides with masses up to 2000 Da, the molecular species with masses higher than this limit were not investigated.

Sequencing of the isoforms

Peptides with relevant molecular masses and strong intensities in the mass spectra were submitted to sequencing using CID in both ESI-Q/ToFMS and MALDI-ToF/ToFMS instruments, and PSD in the MALDI-ToF/ToF instrument. The fragmentation spectra and assigned amino acid sequences of some peptides are shown in Figs. 2 and 3.

Table 1. Mass fingerprinting of fractions containing peptides of interest. Shaded values were submitted to MS/MS sequencing by ESI-Q-ToF. Values shown in bold font were submitted to MS/MS sequencing using both ESI-Q-ToF and MALDI-ToF-ToF

MM (Da)	MM (Da)	MM (Da)	MM (Da)
301.31	1209.74	**1610.12**	3688.62
319.33	1214.80	1611.32	4117.03
335.30	1217.00	1623.92	4119.74
464.44	1232.79	1626.36	4130.92
481.47	1309.88	**1637.93**	4134.05
483.48	1328.74	**1653.94**	4421.64
497.47	1329.03	1660.44	4435.59
503.47	1329.52	1696.39	4449.60
519.46	**1337.89**	1767.47	4916.11
637.65	1348.92	1777.49	4916.27
871.70	1376.93	1922.61	5493.02
955.98	1377.14	1938.94	5510.34
999.88	1434.03	1951.09	5743.22
1027.95	1461.95	1963.65	5942.44
1065.85	1462.25	1966.13	6117.73
1146.86	1480.51	3272.19	6271.51
1169.75	1490.04	3276.74	6554.50
1174.80	1492.96	3566.82	7526.15
1196.94	1509.87	3672.18	7543.18
1200.74	1593.10	3672.88	

The assigned sequences are listed in Table 2. A total of 15 isoforms with nominal masses in the range 871–1654 Da, containing 7–14 amino acid residues, were found. These are linear peptides with some particular features, one of them being the cyclization of the N-terminal glutamine to pyroglutamic acid which is common to all of the isoforms. They also have a common scaffold composed of basic and acidic amino acids (PyrKKDKKDx, where x can be either K or R), which stretches from the first to the eighth residue and is conserved in almost all of the determined isoforms. Two observed exceptions to this common scaffold were shown by isoforms with either the mutation (K3N) or deletion (K5K6D7 or K5) sites. Interestingly, the appearance of the isoforms lacking the sequence K5K6D7 could indicate that this scaffold is created by either an alternative splicing or by the repetition of the three codons that code for this portion.

Another important feature of these peptides is located near their C-termini. Most of the observed sequences finish with a hydrophobic tail composed of 5–6 residues, in which the last residue is amidated. However, in some of the observed isoforms, this C-terminal motif is completely lacking or contains only one or two aromatic residues (i.e., F or FY) and, in addition, are non-amidated. It is possible, therefore, that the absence of this hydrophobic tail in some of the isoforms is the result of proteolytic degradation unrelated to the maturation of the peptide. Importantly, the C-terminal residues of all those peptides containing the hydrophobic tail were found to be amidated. This observation is consistent with a specific maturation of the peptide, since it is known that the amidation of a given C-terminal residue in toxins is a result of the cleavage of the precursor chain by an α-amidating enzyme, as verified for scorpion toxins.[10,11]

A search of databanks performed by FASTA3[12] (see also the Internet[13]) resulted in an 80% of identity sequence with sialokinin I, a peptide from the yellowfever mosquito *Aedes aegypti*[14] (SwissProt entry P42634), and other peptides belonging to the tachykinin family. A search for the tachykinins keyword at SwissProt[15] revealed 62 entries distributed among many different groups in the phylogenetic scale. Table 3 lists some of the highest similarities of PnTkP peptides with peptides from echiura, molluscs, arthropods, fish, amphibians, birds and mammals. Tachykinins are a ubiquitous family of biologically active peptides, with neurohormonal and vasodilatory activities. They share a conserved C-terminal sequence $FXGLM_{NH2}$, where X is any aromatic or branched aliphatic amino acid residue. In light of these similarities, the present peptides were named *Phoneutria nigriventer* tachykinin peptides I to XV (PnTkP-I to PnTkP-XV). The presence of the PhTkPs in the fraction PhM would undoubtedly account for its smooth muscle-contracting activity,[2,3] and might also to some degree account for the hyperalgesia and oedema effects observed for the crude venom.[16,17] This is because both of these effects seem to be partly mediated by peripheral tachykinin receptors. Further characterization of the biological activity of these peptides, together with investigations of the functional involvement of

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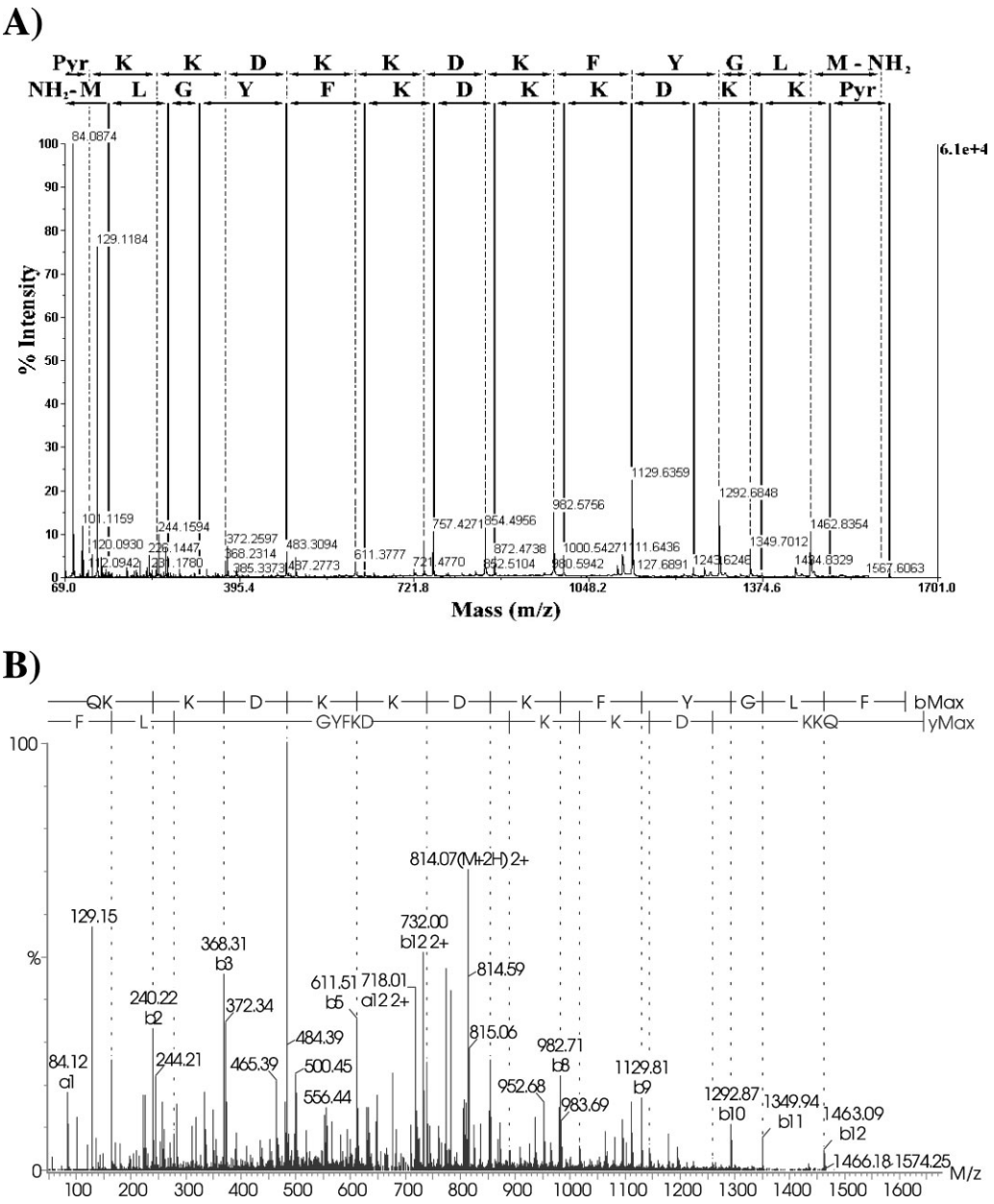

Figure 2. Amino acid sequences of two *P. nigriventer* tachykinin peptides (PnTkP X and PnTkP XII) assigned by MALDI-ToF/ToF and ESI-Q-ToF fragmentation. (A) Fragment ion spectrum obtained by CID with MALDI-ToF/ToFMS for isoform PnTkP X. The precursor ion was *m/z* 1610.88 in charge state 1. (B) Fragment ion spectrum obtained by CID with ESI-Q-ToFMS for isoform PnTkP XII. The precursor ion was *m/z* 814.07 in charge state 2. Fragmentation was achieved using argon as collision gas, with collision energy ramped between 30 and 50 eV. The Pyr residue is shown as Q.

the observed post-translational modifications, are in progress using synthetically assembled peptides, and will be published elsewhere.

A previous work by some of us described the presence of a peptide whose sequence KDKKDCFYGLM was obtained by automated Edman degradation.[5] Attempts to find this isoform in our mass fingerprinting database by searching for the calculated mass (1345.65 Da) failed to find a matching value. It is possible that the C6 residue, originally assigned by a blank cycle in the Edman sequencing data for this peptide, was misidentified. However it is noteworthy that, with the exception of the assigned C6 residue and the absence of the two first amino acid residues, this sequence is identical to that of two of the isoforms described here (PnTkP-X and PnTkP-XIII). Other attempts to sequence these peptides by Edman degradation have failed due to blockage of their N-termini, although, in some cases, the conserved sequence KDKKD was verified.[5]

Assessments of the post-translated N- and C-terminal residues

Assignments of the N- and C-terminal residues in MS/MS sequencing is best achieved through identification of the b1 and y1 fragment ions, respectively. In some cases these ions are not observed, and therefore these assignments are made through the identification of pairs of residues forming the b2 (first plus second residues) and y2 (ultimate plus penultimate residues) ions.

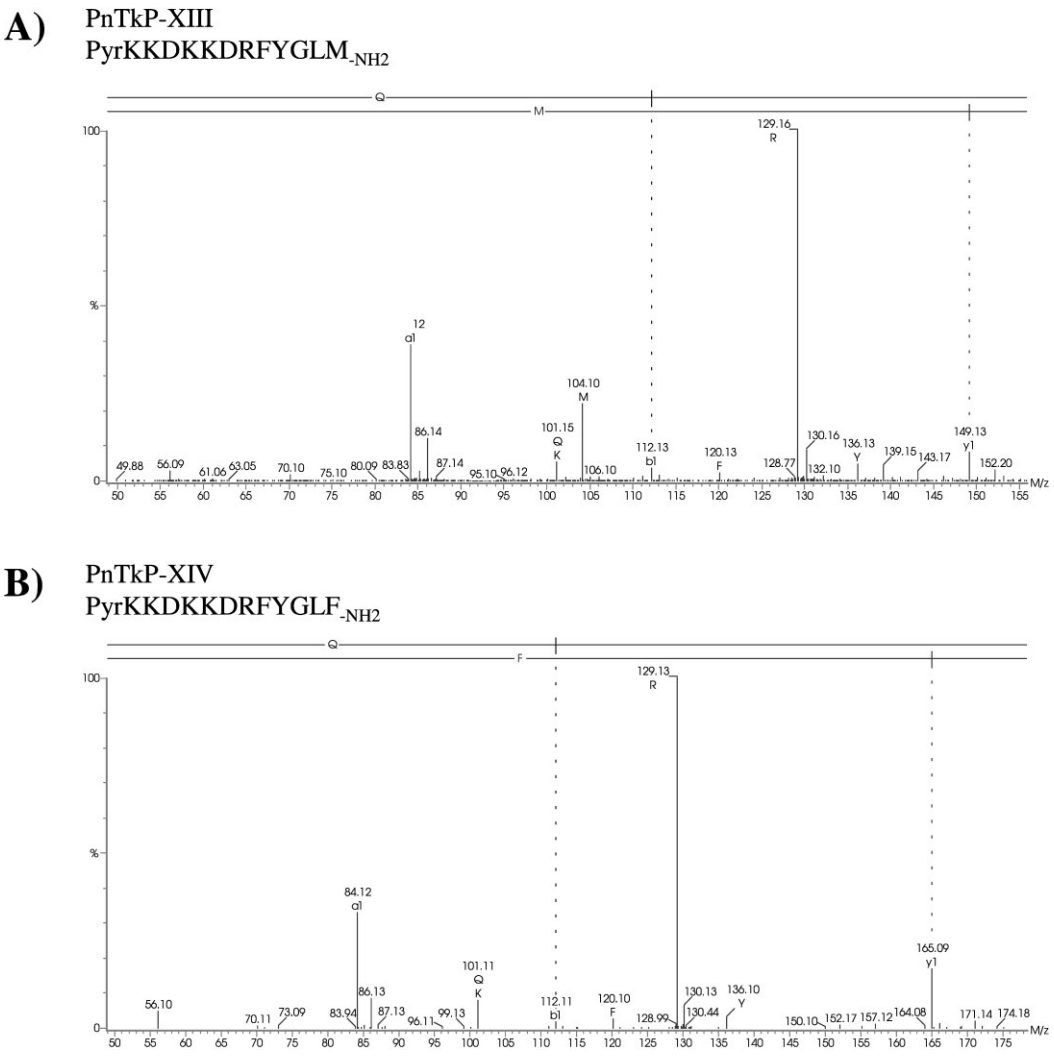

Figure 3. Assignment of modified N- and C-terminal residues. Fragment ions corresponding to the N- and C-terminally modified residues (b1 and y1) of isoforms PnTkP-XII (A) and PnTkP-XIV (B) assigned by CID with ESI-Q-ToFMS. The Pyr residue is shown as Q.

In the present work most of the b1 and all the y1 ions could be positively verified, as indicated in Table 2. Cyclization of Gln residues to form the pyroglutamic acid residue (Pyr) was verified in most of the sequenced peptides by the presence of a b1 (*m*/*z* 112) ion (Table 2 and Figs. 2 and 3). An ion corresponding to the protonated dipeptide Pyr-Lys (b2) was observed at *m*/*z* 240 for all of the sequenced peptides (Table 2).

The C-terminal residues of some of the isoforms were found to be amidated, and this was found to be the case for all those peptides containing a hydrophobic tail composed of 5 or 6 hydrophobic residues. Such tails might be terminated by Met, Phe or Val residues. Therefore, the ions at *m*/*z* 149, 165 and 117, respectively, were assigned as the corresponding y1 ions (Table 2 and Figs. 2 and 3).

Enzymatic cleavage and processing of peptides

All the peptides observed in this work underwent a C-terminal cleavage processing which seems to occur in different manners for different cases. The question arises as to whether these processing cleavages were all related to the maturation of the peptides, or whether some of them were the results of enzymatic degradation after the venom extraction. It seems reasonable to assume that the mature processed peptides are those whose sequences are C-terminated by an amidated residue (Table 2). On the other hand, sequences that have non-amidated C-terminal residues seem to be the result of enzymatic degradation by trypsin and/or chymotrypsin-like enzymes, which are present in this venom (unpublished data). These enzymes may not be constituents of the secreted venom, and instead could be contaminants from gastric fluids that are occasionally present in the venom obtained by electrical milking. In fact, it is common to find these kinds of cleavages in the other toxins present in this venom and which have their fragments held together by disulfide bridges (unpublished data).

It has also been previously observed that the muscle activity described for fraction PhM is destroyed by incubation with chymotrypsin.[3] On consideration of these observations together with the present sequence data, it is most likely that the hydrophobic tail is somehow important for the biological activity of these peptides.

CONCLUSIONS

From the evolutionary point of view, toxin isoforms found in animal venoms can be very important by acting synergistically once inside the victim's body. Furthermore, isoforms

Table 2. Identification of post-translationally modified isoforms from *P. nigriventer* venom

	Peptides identified			N- and C-terminal fragments (*m/z*)			
Name	Sequence	$MM_{(Calc)}$[a]	$MM_{(Obs)}$	b1	b2	y1	y2
PnTkP-I	PyrKKDKKD	871.48	871.70	112.12	240.20	134.08	262.21
PnTkP-II	PyrKKDKKDK	999.57	999.88	nd	240.21	147.16	262.22
PnTkP-III	PyrKKDKKDR	1027.58	1027.95	112.13	240.23	175.19	290.25
PnTkP-IV	PyrKKDKKDKF	1146.64	1146.86	112.09	240.19	166.11	294.24
PnTkP-V	PyrKKDKKDRF	1174.65	1174.80	112.11	240.18	175.15	322.25
PnTkP-VI	PyrKKD---RFL–GLM$_{-NH2}$	1216.68	1217.00	nd	240.20	149.13	262.27
PnTkP-VII	PyrKKD---RFL–GLF$_{-NH2}$	1232.70	1232.79	112.12	240.16	165.09	nd
PnTkP-VIII	PyrKKDKKDRFY	1337.71	1337.89	112.10	240.16	182.11	329.17
PnTkP-IX	PyrKKDK–DRFY–GLM$_{-NH2}$	1509.78	1509.87	112.11	240.15	149.10	262.18
PnTkP-X	PyrKKDKKDKFY–GLM$_{-NH2}$	1609.87	1610.12	112.10	240.17	149.10	262.19
PnTkP-XI	PyrKNDKKDRFY–GLM$_{-NH2}$	1623.81	1623.92	112.11	240.21	146.13	262.18
PnTkP-XII	PyrKKDKKDKFY–GLF$_{-NH2}$	1625.89	1626.36	nd	240.22	165.13	278.25
PnTkP-XIII	PyrKKDKKDRFY–GLM$_{-NH2}$	1637.87	1637.93	112.11	240.15	149.09	262.16
PnTkP-XIV	PyrKKDKKDRFY–GLF$_{-NH2}$	1653.89	1653.83[c]	112.11	240.16	165.09	278.19
PnTkP-XV	PyrKKDKKDRFPNGLV$_{-NH2}$	1653.93	1653.94[c]	112.09	240.14	117.10	230.19
PhM1[b]	KDKKDCFY–GLM$_{-NH2}$	1345.65	nd	nd	nd	nd	nd

Pyr: Pyroglutamic acid from glutamine. NH_2: C-terminally amidated peptide.
[a] Molecular mass calculated as monoisotopic mass (Da).
[b] PhM1 peptide: sequence obtained by Edman degradation.[5]
[c] Masses assessed by both ESI-Q-ToF and MALDI-ToF/ToF. Underlined L means no distinction between Leu or Ile. Bold **L** was assigned by similarity with Edman-sequenced peptide.

Table 3. Alignment of some similar sequences of the tachykinin family with some of the PnTkPs

Peptide	Sequences	$MM_{(Calc)}$[a]	Organism	Accession no.[b]	Length
PnTkP–VI	PyrKKD---RFL–GLM$_{NH2}$	1216.68	*P. nigriventer*	—	10
PnTkP–VII	PyrKKD---RFL–GLF$_{NH2}$	1232.70	*P. nigriventer*	—	10
PnTkP–IX	PyrKKDK–DRFY–GLM$_{NH2}$	1509.78	*P. nigriventer*	—	12
PnTkP–X	PyrKKDKKDKFY–GLM$_{NH2}$	1609.87	*P. nigriventer*	—	13
PnTkP–XI	PyrKNDKKDRFY–GLM$_{NH2}$	1623.81	*P. nigriventer*	—	13
PnTkP–XII	PyrKKDKKDKFY–GLF$_{NH2}$	1625.89	*P. nigriventer*	—	13
PnTkP–XIII	PyrKKDKKDRFY-GLM$_{NH2}$	1637.87	*P. nigriventer*	—	13
Sialokinin I	--N–TGDKFY–GLM$_{NH2}$	1126.51	*Aedes aegypti* (Yellowfever mosquito)	P42634	10
Physalaemin	Pyr–AD–PNKFY–GLM$_{NH2}$	1264.59	*Physalaemus fuscumaculatus* (Neotropical frog)	P08610	10
Phyllomedusin	Pyr–N--PNRFI–GLM$_{NH2}$	1170.60	*Phyllomedusa bicolor* (Two-colored leaf frog)	P08615	11
Eledoisin	Pyr--PSKDAFI–GLM$_{NH2}$	1187.60	*Eledone moschata* (Musky octopus)	P01293	11
Scyliorhinin I	-----AKFDKFY-GLM$_{NH2}$	1200.60	*Scyliorhinus canicula* (Spotted dogfish)	P08608	10
Neurokinin A	-----HKTDSFV-GLM$_{NH2}$	1115.54	*Homo sapiens* (Human)	P20366	10
Neurokinin A	-----HKTDSFV-GLM$_{NH2}$	1115.54	*Gallus gallus* (Chicken)	P19851	10
PnTkP-XIV	PyrKKDKKDRFY-GLF$_{NH2}$	1653.89	*P. nigriventer*	—	13
PnTkP-XV	PyrKKDKKDRFPNGLV$_{NH2}$	1653.93	*P. nigriventer*	—	14
Urechistachykinin I	---LRQSQFV-GSR$_{NH2}$	1158.62	*Urechis unicinctus* (Echiuroid worm)	P40751	10

Pyr: Pyroglutamic acid from glutamine. NH_2: C-terminally amidated peptide.
[a] Molecular masses calculated as monoisotopic masses (Da).
[b] SwissProt entry.[15]

can also represent an evolutionary countermeasure against the immunological system of the prey. On the other hand, for the purposes of toxinological studies, we can use these natural mutants and post-translated peptides in structure-function studies to better understand the mode of actions of these toxins. Specifically in the PnTkP family, structure-function studies can reveal how important the observed modifications are for their function, and what are the weak points in the amino acid chain that are initially subjected to (or are more exposed to) enzymatic degradation.

Mass spectrometric analyses with reasonably high resolution and mass accuracy, followed by sequencing using mass spectrometry, have made it possible to solve the structures of these peptides. Chemical synthesis is now required in order to gain enough material for work on the characterization of the biological activity of these peptides.

Although this work represents a step toward a deeper characterization of the primary structures of the peptides from the venom of *Phoneutria nigriventer*, a full comprehension of the puzzling complexity of this venom is far from complete. Some of the molecular ions identified by the sensitive ESI-Q/ToFMS and MALDI-ToF/ToFMS analyses are still of too low abundance to be confidently sequenced. This difficulty arises at least in part due to signal suppression by the more abundant molecules. This suggests that further improvements in fractionation using capillary systems are required to enhance these signals. It is also probable that some of these small molecules do not represent mature peptides, but are instead the result of precursor processing and/or degradation.

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