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Comparison of the partial proteomes of the venoms of Brazilian spiders of the genus *Phoneutria* ☆

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Abstract

The proteomes of the venoms of the Brazilian wandering "armed" spiders *Phoneutria nigriventer*, *Phoneutria reidyi*, and *Phoneutria keyserlingi*, were compared using two-dimensional gel electrophoresis. The venom components were also fractionated using a combination of preparative reverse phase HPLC on Vydac C4, analytical RP-HPLC on Vydac C8 and C18 and cation exchange FPLC on Resource S at pH 6.1 and 4.7, or anion exchange HPLC on Synchropak AX-300 at pH 8.6. The amino acid sequences of the native and S-pyridyl-ethylated proteins and peptides derived from them by enzymatic digestion and chemical cleavages were determined using a Shimadzu PPSQ-21^{A} automated protein sequencer, and by MS/MS collision induced dissociations. To date nearly 400 peptides and proteins (1.2 – 27 kDa) have been isolated in a pure state and, of these, more than 100 have had their complete or partial amino acid sequences determined. These sequences demonstrate, as might be expected, that the venoms of *P. reidyi* and *P. keyserlingi* (Family: Ctenidae) both contain a similar range of isoforms of the neurotoxins as those previously isolated from *P. nigriventer* which are active on neuronal ion (Ca^{2+}, Na^{+} and K^{+}) channels and NMDA-type glutamate receptors. In addition two new families of small (3 – 4 kDa) toxins, some larger protein (>10 kDa) components, and two serine proteinases of the venom of *P. nigriventer* are described. These enzymes may be responsible for some of the post-translational modification observed in some of the venom components.

Keywords: Enzymes; Insecticides; Neurotoxins; *Phoneutria*; Proteomes; Spiders; Venoms; Amino acid sequences; Sequence similarities

1. Introduction

Spiders such as those belonging to the genus *Phoneutria* are an important part of the rich biodiversity which may be found in Brazil. Their venoms contain a wide variety of proteins and peptides, including neurotoxins which act on the ion channels and chemical receptors of the neuro-muscular systems of insects and mammals. These venoms have been described as a treasure chest for the future discovery and development of new biologically active molecules with potential application in medicine and agriculture (Escoubas et al., 2000; Gomez et al., 2002; Rash and Hodgson, 2002).

The very aggressive South American solitary "armed" or "wandering" spider *Phoneutria nigriventer* (Keys.) is responsible for most human accidents of araneism, including the death of infants, in Central and Southern Brazil (Lucas, 1988). Early studies revealed that its venom contained potent neurotoxins which caused excitatory symptoms such as

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salivation, lachrymation, priapism, convulsions, flaccid and spastic paralysis of the anterior and posterior members, and death following intracerebral injection in mice (Diniz, 1963; Schenberg and Pereira Lima, 1971; Entwhistle et al., 1982). Subsequently three groups of fractions of neurotoxins (PhTx1, PhTx2 and PhTx3) and a non-toxic fraction which had activity on smooth muscle were purified from the venom (Rezende et al., 1991). Later a fourth fraction (PhTx4) was isolated which was extremely toxic to insects of the orders Diptera and Dictyoptera, but had much weaker toxic effects on mice (Figueiredo et al., 1995).

From these fractions the complete amino acid sequences were determined for toxin Tx1 (Diniz et al., 1990), four Tx2 toxins (Cordeiro et al., 1992), six of the Tx3 type (Cordeiro et al., 1993), two of the non-toxic smooth muscle active group (Cordeiro et al., 1995) and three of the insecticidal Tx4 type (Figueiredo et al., 1995, 2001; Oliveira et al., 2003). The primary structures of most of these molecules were subsequently confirmed by the analyses of clones from cDNA libraries constructed using the venom gland of the spider (Diniz et al., 1993; Kalapothakis et al., 1998a,b; Kushmerick et al., 1999; Penaforte et al., 2000; Matavel et al., 2002).

Parallel pharmacological and electrophysiological studies on these purified venom peptides have revealed that Tx1 acts on Ca^{2+} channels (Santos et al., 1999), toxins of the type Tx2 affect Na^{+} channels (Araujo et al., 1993a,b; Matavel et al., 2002; Yonamine et al., 2004), and the Tx3 group acts on Ca^{2+} or K^{+} channels (Troncone et al., 1995; Prado et al., 1996; Guatimosim et al., 1997; Leão et al., 2000; Miranda et al., 1998, 2001; Cassola et al., 1998; Kushmerick et al., 1999; Gomez et al., 2002; Santos et al., 2002; Vieira et al., 2003; Carneiro et al., 2003). The insecticidal toxin Tx4(6-1) stimulated glutamate release at neuromuscular junctions in cockroach (Figueiredo et al., 1997) and slowed down Na^{+} current inactivation in insect central nervous systems (CNS), but was ineffective on mammalian Na channels (De Lima et al., 2002). Despite their apparent lack of toxicity for mammals, the insecticidal PhTx4 class of toxins were shown to inhibit glutamate uptake in rat brain synaptosomes (Mafra et al., 1999), with Tx4(5-5) selectively and reversibly inhibiting the *N*-methyl-D-aspartate (NMDA) sub-type of ionotropic glutamate receptors in rat hippocampal neurones (Figueiredo et al., 2001). In addition to these studies on the purified toxins, other workers have reported on the various pharmacological and electro-physiological effects of the whole (crude) venom or partially purified fractions from *P. nigriventer* (Estato et al., 2000; Costa et al., 2000, 2001, 2002; Weinberg et al., 2002; Le Sueur et al., 2003; Zanchet and Cury, 2003; Teixeira et al., 2004).

Until recently, all studies on the venom of *Phoneutria* have been restricted to the species *P. nigriventer* and mostly confined to peptides in the size range of 3.5 kDa to 9 kDa. However, we have recently described the existence of a highly complex pool of smaller (<2 kDa) peptides that provoke contractions in the smooth muscle of guinea pig ileum. The amino acid sequences of 15 isoforms were determined by tandem mass spectrometry (MS/MS) using both electrospray ionization quadrupole time of flight spectroscopy (ESI-Q/ToFMS) and matrix-assisted laser desorption/ionization tandem time of flight mass spectroscopy (MALDI-ToF/ToFMS) (Pimenta et al., 2005). All of these molecules which are structurally related to the tachykinin family of neuro-hormone peptides possessed N-terminal pyroglutamate residues and exhibited evidence of other post-translational modifications such as proteolysis and C-terminal amidation.

In this present work we now describe several other new families of small (3–4 kDa) toxins and some larger protein (>10 kDa) components of the venom of *P. nigriventer*. In addition we report for the first time the structures of 30 new proteins purified from the venoms of the two related species of spiders *Phoneutria reidyi* and *Phoneutria keyserlingi*.

2. Materials and methods

2.1. Venoms

Male and female specimens of the spiders *P. nigriventer* (Keys.) and *P. keyserlingi* were collected in the regions of Santa Barbara and Mariana, respectively, both in the State of Minas Gerais, and kept in the arachnidarium of the Fundação Ezequiel Dias (Belo Horizonte, Brazil). Venom from the live adult spiders was obtained by electrical stimulation of the fangs as described by Barrio and Vital Brazil (1949). The venom (5–12 μL/spider, 160 mg/mL) was immediately transferred to siliconized glass tubes in ice, diluted with the same volume of distilled water and centrifuged at 4000×*g* to remove insoluble materials and cellular debris. The supernatant was lyophilized and stored at −18 °C. The venom of the spider *P. reidyi* which was collected using the above method from specimens captured in the vicinities of the hydro-electric reservoirs at Tucurui (State of Para), Samuel (State of Roraima) and Balbina (Amazonas) was a generous gift from the Butantan Institute (São Paulo, Brazil).

2.2. Two-dimensional electrophoresis

Immobiline DryStrips (11 cm; pH 3–10, Amersham) were rehydrated overnight with rehydration buffer (7 M urea, 2 M thiourea, 1% triton X-100, 0.5% Pharmalyte 3–10, 65 mM 1,4-dithio-DL-threitol (DTT)) containing approximately 300 μg of the venom proteins. Running was performed in a Multiphor II Isoelectric focusing (IEF) system from Amersham Pharmacia Biotech. Electrical conditions were as described by the supplier. After the first-dimensional run, the IPG gel strips were sealed in plastic wrap and frozen at −80 °C or incubated at room temperature in 3 mL of equilibration buffer (50 mM Tris, 6 M urea, 2% SDS and traces of bromophenol blue) containing 57.8 mg of DTT prior to separation in the second dimension. The second dimension electrophoresis was performed in a vertical system with uniform 15% separating gel (14×14 cm), at 25 °C. Protein spots in the 2-DE gels were visualized by using 0.1% PhastGel Blue R-350 as the stain.

2.3. Purification of venom peptides and proteins

The venoms of all three species were processed in the same manner. Aliquots of 25–30 mg of lyophilized venom were dissolved in 2 mL of aqueous 0.1% trifluoroacetic acid (TFA) and centrifuged at 4000×*g* for 10 min to remove insoluble materials. The brownish yellow supernatant was applied to a preparative column (2.2×25 cm) of Vydac C4 (214TP1022) equilibrated with 0.1%TFA in water(solvent A). Solvent B was 100% acetonitrile containing 0.1%TFA. The column was eluted at a flow rate of 5 mL/min with the following gradient system: 0 to 20 min, 100%A; 20 to 30 min, 0–20%B; 30 to 110 min; 20–40%B; 110 to 130 min, 40–50%B; 130 to 150 min, 50–70%B. The presence of peptides or proteins in the eluate was detected by measuring the UV absorption at 214 nm. Fractions containing peptides or proteins were collected manually and lyophilized.

The lyophilized fractions obtained from the preparative reverse phase HPLC(RP-HPLC) were then dissolved in 2 mL of 10 mM sodium phosphate buffer pH 6.1 and subjected to ion-exchange FPLC on a column (6.4 mm×30 mm) of Resource™ S (Amersham Pharmacia Biotech) equilibrated in the same buffer. After application of the sample, the column was initially washed with the starting buffer for 10 min and then eluted with a gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 1 mL/min over a period of 45 min. Polypeptides were detected by absorbance at 214 nm. Fractions which still required further purification or did not bind to the cation exchanger buffered at pH 6.1 were reapplied to the same column buffered at pH 4.3 with 10 mM sodium acetate and eluted with a gradient of 0–0.5 M NaCl in the same buffer. A small number of fractions from the preparative RP-HPLC step which were not well resolved by using cation exchange chromatography were fractionated on an anion exchange HPLC column (4.1 mm×30 cm) of Synchropak AX-300 (Synchrom Inc.) using a linear gradient of 0–0.5 M NaCl in 10 mM Tris–HCl buffer pH 8.6 at a flow rate of 1 mL/min.

The venom components obtained from these cation and anion exchange FPLC and HPLC steps were desalted and in some cases further purified by RP-FPLC or RP-HPLC on analytical columns of PepRPC™(15 um, HR10/10, Pharmacia LKB), Vydac C8 or C18 using various extended gradients of acetonitrile in 0.1% TFA. The homogeneity (purity) of all the fractions obtained was examined by PAGE and mass spectroscopy as described below.

2.4. Bioassays

The toxicity of each purified peptide/protein was assayed qualitatively on five house flies (*Musca domestica*, 16–20 mg, 3-day-old) and two albino mice (*Mus musculus*) (18–22 g). The lyophilized samples were dissolved in 0.15 M saline, containing 0.25 mg/mL of bovine serum albumin (BSA), for injection. House flies previously restrained by chilling at 4 °C were injected in the thoracic body cavity. The injections were performed with fine capillaries made from micropipettes, inserted on a flexible tube attached to a 10 or 25 μL Hamilton syringe delivering, respectively, a volume of 0.5 or 1.0 μL containing known amounts of each fraction. Control animals received physiological saline alone. The following signs of intoxication were assessed: excitability, salivation, trembling of the legs and body, jerking of the limbs, knock-out, loss of ability to walk or fly and death. The effects in mice were assayed by icv (intracerebroventricular) injection of 5 μL of the samples, as described by Rezende et al. (1991). The appearance of neurotoxic symptoms (excitation, salivation, lachrymation, priapism, spastic or flaccid paralysis, scratching, tail elevation) or the death of animals was observed after injection. Control animals received physiological saline alone.

For the crude (whole) venoms the median-lethal dose (LD_{50}) was calculated by probit analysis (Finney, 1964), of the results obtained following the injections of the venoms at 6 different dose levels, using 12 mice and 15 house flies at each dose level.

2.5. Electrophoresis

Propionic acid/urea-PAGE was performed according to the method previously described by Chettibi and Lawrence (1989) with 22.5% gels. Tricine-SDS-PAGE was carried out as described by Schagger and von Jagow (1987), using gels that were composed of a small-pore gel of 16.5%T–3%C, overlaid by a 4%T–3%C stacking gel.

2.6. Proteolytic activity assays

The presence of gelatinolytic activity in various fractions was detected by zymography as described by Heunssen and Dowdle (1980) using SDS-PAGE-gelatin. Electrophoresis was carried out using 7.5% gels containing 0.1% gelatin. After electrophoresis, the gel was rinsed in 2.5%(v/v) of Triton X-100 for 1 h in order to remove SDS and then incubated overnight at 37 °C in buffers of different pH values. In this method the proteolytic activity on gelatin is detected as colorless bands on the otherwise blue gel after staining with coomassie blue.

The caseinolytic activity of fractions was measured with succinylated casein as substrate in conjunction with trinitrobenzenesulfonic acid using the QuantiCleave™ Protease assay kit (Pierce, Rockford, IL, USA).

2.7. Mass spectrometry analyses

ES-Q-TOF mass spectrometry analyses were carried out using a Q-TOF Micro™ Micromass, UK) equipped with an electrospray ionization source operated in positive mode. Capillary voltage was 3000 V and sample cone voltages were 40–60 V. Mass spectrometer calibrations were made by using sodium iodide with caesium iodide in 2000 Da range. Samples diluted in 50% acetonitrile/ 0.1% TFA were introduced by using a syringe pump with flow rates of 5–10 μL/min. The spectrum used was the result from 20 scans (2.4 s) combined. Original data (*m*/*z*) were treated (base line subtraction, smoothing and centering) and transformed into a mass (Da)

spectrum. Collision induced dissociation (MS/MS) was carried out using argon and collision energies in the range 30–45 V. Data analyses were carried out using MassLynx® 3.5 software.

2.8. S-reduction and alkylation

The peptides/proteins (20–200 nmol) were S-reduced and alkylated with vinyl pyridine essentially as described by Henschen (1986). The material was dissolved in 1 mL of 6 M guanidine–HCL in 0.1 M Tris–HCl, pH 8.6. To this solution 30 µL of 2-mercaptoethanol (pure liquid 14.3 M) was added under nitrogen, and the sample incubated at 50 °C for 4 h. After this, 40 µL of vinyl pyridine (95%) was added and the samples were incubated at 37 °C for a further 2 h. The reduced and alkylated peptides/proteins were recovered by desalting on a column (22 mm × 25 cm) of Vydac C4 (214TP54), using a gradient of 0 to 70% acetonitrile in 0.1% TFA over 70 min at a flow rate of 1 mL/min. The collected materials were lyophilized.

2.9. Determination of amino acid sequences

Samples of the S-pyridyl-ethylated proteins were dissolved in 1 mL of 0.1 M ammonium bicarbonate pH 7.9 and digested separately at 37 °C with trypsin (for 3.5 h), chymotrypsin (for 4 h) and the GLU-specific endoproteinase from *Staphylococcus aureus* V8(for 18h) using 2%(w/w) enzyme/substrate. Other protein samples were treated with a 500- fold molar excess of cyanogen bromide in 70% TFA under nitrogen for 24 h to achieve cleavage of Met-X peptide bonds as described by Aitken et al. (1989). Cleavage at Trp-X peptide bonds was carried out using *o*-iodosobenzoic acid as described by Aitken et al. (1989). After lyophilization the peptides produced were separated by RP-HPLC on a column (4.6 mm × 25 cm) of Vydac C18 (small pore, 201SP54) using an extended gradient of 0 to 50% acetonitrile in 0.1% TFA for 180 min at a flow rate of 1 mL/min. Certain peptides which failed to sequence when subjected to Edman degradation were unblocked by treatment with pyroglutamate aminopeptidase in 50 mM Na phosphate buffer pH 7.0 containing 10 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid (EDTA) at 50 °C for 6 h.

The amino acid sequences of the S-pyridyl-ethylated intact proteins (2–10 nmol) and the peptides derived from them by the enzymatic digestions were determined by Edman degradation using a Shimadzu PPSQ-21^{A} automated protein sequencer.

2.10. Sequence comparisons

The amino acid sequences of the various peptides/proteins were compared with the sequences of other related proteins in the SWISS-PROT/TREMBL data bases using the FASTA 3 and BLAST programs.

2.11. Protein nomenclature

Each protein/peptide purified and studied during this work is described by a code. For example the species of *Phoneutria* from which it originated is indicated by PN = *P. nigriventer*; PR = *P. reidyi*, or PK = *P. keyserlingi*. The first number indicates the number of the peak eluted from the preparative RP-HPLC on Vydac C4 which contained the protein. The following letter C or A indicates whether the second step was cationic exchange FPLC (Resource S, pH 6.1) or anionic exchange HPLC (Synchropak AX300, pH 8.6). The subsequent number indicates the peak number during the second step. In a few cases, when a third step of ion exchange chromatography (C or A) was required, this is indicated using the same logic. We have maintained the original nomenclature (e.g. Tx1, Tx3-1, Tx4(6-1), etc.) for those peptides/proteins previously described by us (Diniz et al., 1990; Cordeiro et al., 1992, 1993; Figueiredo et al., 1995, 2001).

3. Results and discussion

When the crude venoms of each of the three species of *Phoneutria* spiders were subjected to 2D-PAGE, and the resulting gels stained with Fast gel blue, approximately 80 spots were visible in each case (Fig. 1). It is difficult to assess the total number of peptide/protein components in each venom with great accuracy as there are clearly several overlapping spots in those regions of the gels containing the cationic proteins with molecular masses of <12 kDa, and also peptide

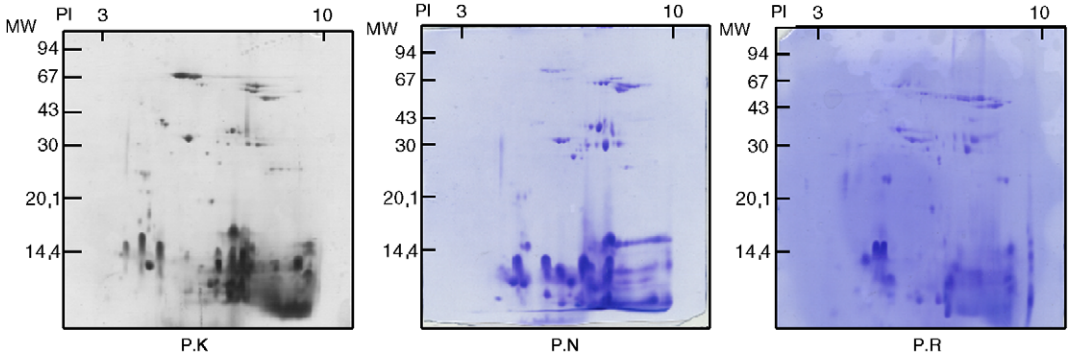

Fig. 1. Comparison of the two-dimensional gel electrophoresis patterns obtained for the crude venoms (mixtures obtained from both male and female spiders) of *Phoneutria keyserlingi* (PK), *Phoneutria nigriventer* (PN) and *Phoneutria reidyi* (PR). The protein load of crude venom in each gel was approximately 300 µg. The gels were not subjected to any image processing after destaining.

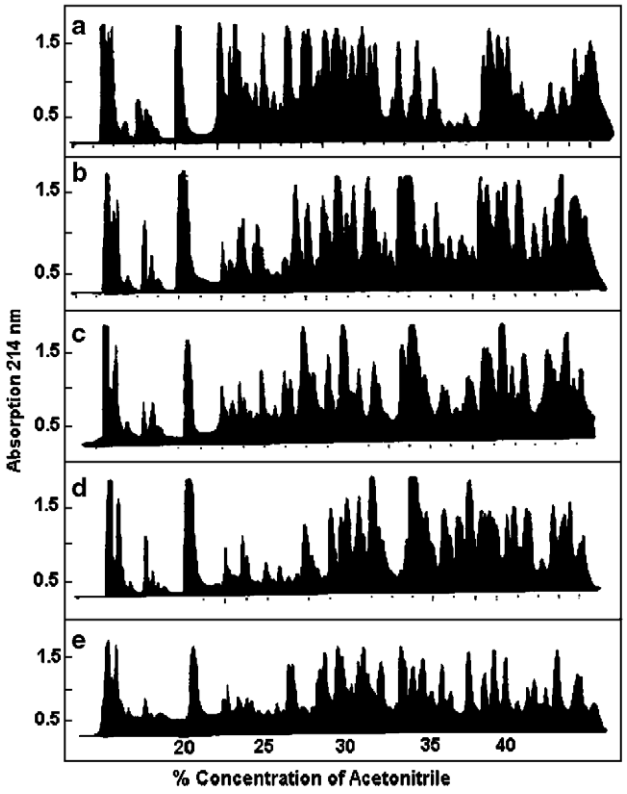

Fig. 2. Comparison of the preparative reverse phase HPLC (Vydac C4) profiles obtained for the crude venoms (mixtures obtained from both male and female spiders) of (a) *Phoneutria nigriventer*, (b) *Phoneutria reidyi* from Amazonas, (c) *P. reidyi* from Roraima, (d) *P. reidyi* from Para, and (e) *Phoneutria keyserlingi*. The amount of protein injected onto the column was 30 mg for panels (a)–(d), and 15 mg in the case of panel (e). The presence of peptides or proteins in the eluate was detected by measuring the UV absorption at 214 nm. All other experimental details are given in the text.

components with masses below 4 kDa are very poorly resolved by this technique. Furthermore we have recently reported that an initial fingerprinting by RP-HPLC/mass spectroscopy revealed that a total of 79 main molecular species were present in a fraction of the venom of PN containing components with molecular masses in the range of 301.31–7543.18 Da (Pimenta et al., 2005). Therefore it seems likely that the total number of peptides and proteins in each venom is above 150. This comparison of the crude venoms of the different species of *Phoneutria* spiders by 2D-PAGE (Fig. 1) revealed some obvious similarities in their compositions. For example in all three venoms the majority of the components are in the size range of 3–12 kDa and are predominantly cationic peptides (pI>7). There are however visible differences in the location and intensity of staining of several components.

During the initial fractionation of the different *Phoneutria* venoms by preparative RP-HPLC, in each case approximately 55 peaks of absorption at 214 nm were obtained (Fig. 2). The peaks numbered 1–7 contained only small peptides (<0.5 kDa) and non-proteinaceous materials, whilst peaks 8–55 contained the larger peptides and proteins. Each of the peaks 8–55 was subsequently purified further by cation exchange FPLC and shown to contain an average of three or more peptides/proteins, which supports the estimate that the venoms of these *Phoneutria* spiders contain more than 150 peptide/protein components. It is interesting to note that in recent preliminary MS analyses, the proteome of the venom of the tarantula *Psalmopoeus cambridgei* (Choi et al., 2004) was also found to contain more than 150 molecular species with masses in the range of 1000–6000 Da, whereas Legros et al. (2004) detected only 65 components in the venom of the tarantula *Theraphosa leblondi*. On the otherhand the scorpion *Tityus serrulatus* contained 380 distinct molecular masses in the toxic fractions alone (Pimenta et al., 2001).

The RP-HPLC results obtained (Fig. 2) also clearly confirmed that although the venoms had an overall similarity in their components, there were both qualitative and quantitative differences between the venoms from the three different species. One of the most obvious differences between the venoms can be seen when the HPLC profiles for *P. nigriventer* (PN) and *P. reidyi* (PR) are compared in the region where the eluting gradient of acetonitrile has a concentration of 32–37%. It appears that the venom of *P. reidyi* contains a much higher concentration of the components which elute in this part of the gradient. We have previously reported that the fractions which elute in this concentration of acetonitrile contain those toxins which are more potent for insects (house flies, crickets and cockroaches) than for mice (Figueiredo et al., 1995, 2001). This correlates well with our observation that the whole venom of *P. reidyi* is approximately 20 times more toxic for insects than the crude venom of *P. nigriventer* (Table 1).

Differences were also visible in the HPLC profiles of the three samples of venom of *P. reidyi* obtained from spiders collected in different geographical regions (Fig. 2b, c, d), However it should be remembered that such differences may be also caused by factors other than geography. For example other workers have reported marked variations in the venom of *P. nigriventer*, examined by SDS-PAGE and RP-HPLC, which were determined by the sex and size/age of the spiders used (Herzig et al., 2002, 2004). This type of intersexual variation was confirmed during this present work when the venoms collected separately from adult male and female spiders of *P. keyserlingi* were compared by 2D-PAGE (Fig. 3). In general the venom from male spiders contains a greater number of components than the venom from females, and this is particularly evident when the proteins with molecular weights of above 25 kDa are examined.

Another possible cause of heterogeneity in venom samples could be the activity of endogenous proteases. Previous

Table 1
Comparison of the toxicities (LD_{50}) of the crude (whole) venoms of spiders of the genus *Phoneutria*

Venom	LD_{50} in mice (μg/20.0±2.0 g)	LD_{50} in flies (ng/20.0±2.0 mg)
P. nigriventer	0.12 (0.10–0.16)	44.80 (35.8–71.3)
P. keyserlingi	0.18 (0.13–0.23)	Nd
P. reidyi	0.22 (0.18–0.26)	1.70 (1.0–2.20)

The median-lethal dose (LD_{50}) values were calculated by probit analysis (Finney, 1964) of the results obtained from injections at six different dose levels, using groups of 12 mice and 15 house flies at each dose level. The values in parentheses are the confidence limits. Nd, values not determined. Other details as described in Materials and methods.

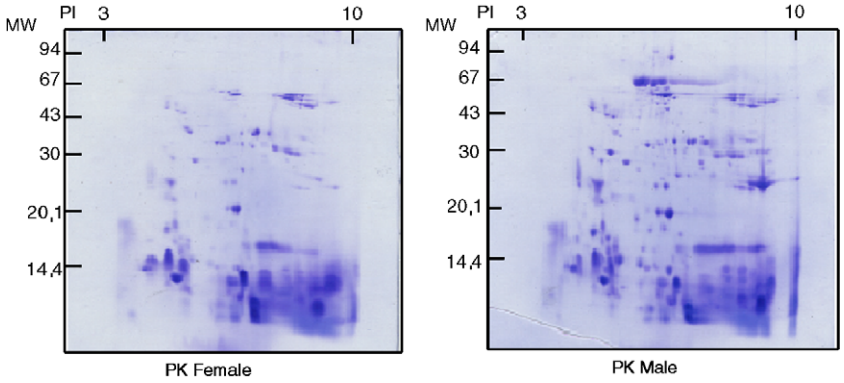

Fig. 3. Comparison of the two-dimensional gel electrophoresis patterns obtained for the crude venoms from separate populations of female and male spiders of the species *Phoneutria keyserlingi* (PK). The protein load in each gel was approximately 300 μg. The gels were not subjected to any image processing after destaining.

workers have reported the presence of proteases in the venom of *P. nigriventer* (Rezende et al., 1991) and other spiders (Shikata et al., 1995), but others have suggested that the enzyme activity is due to contamination with saliva, or the contents of the digestive tract regurgitated during the electrically-stimulated milking process of venom extraction (Perret, 1977; Rekow et al., 1983; Kuhn-Nentwig et al., 1994). During this present work the presence of proteases in the crude venoms and the purified sub-fractions of all three species of *Phoneutria* was demonstrated by SDS-PAGE/gelatin zymography and direct assays using succinyl-casein as the substrate (results not shown).

In all of our early studies on the venom of *P. nigriventer* (Diniz et al., 1990; Cordeiro et al., 1992, 1993; Figueiredo et al., 1995) we routinely employed gel filtration on a long (200 cm) column of Sephadex G-50 as the first step in the purification procedure. This prolonged (24–30 h) chromatography conducted at pH 6.3 and 4 °C has now been omitted from our procedure in an attempt to minimize proteolytic nicking/hydrolysis of peptide bonds in the venom proteins and peptides by any endogenous proteases.

When the crude venom of *P. nigriventer* was subjected to the first preparative RP-HPLC step of purification on Vydac C4, the proteolytic activity was highest in the peaks numbered PN44 to PN49 which eluted with retention times/acetonitrile concentrations of 114 min/ 41%, 117 min/43%, 120 min/45%, 122 min/46%, 124 min/47%, and 126 min/48%, respectively. The major proteins in peaks PN44 and PN47 were subsequently purified to homogeneity by ion-exchange FPLC on Resource S at pH 6.1, followed by rechromatographies on analytical HPLC columns of Vydac C8 and C18 (details not shown). The complete amino acid sequence of PN47 and the N-terminal sequence of PN44 which are shown in Fig. 4, show that both proteins are serine proteases belonging to the peptidase S1 family and have strong (60%) sequence identities with the peptide isomerase previously isolated from the venom of the funnel web spider *Agelenopsis aperta* (Shikata et al., 1995), and lesser similarity with the chymotrypsin-like proteinase from the brown sea squirt *Herdmania momus* (Arnold et al., 1997). Recently our collaborators at the Butantan Institute in São Paulo have demonstrated that these proteases are involved in the development of the hyperalgesia caused by the venom of *P. nigriventer* which is mediated by peripheral tachykinins and excitatory amino acid receptors (Cury et al., 2003; Zanchet and Cury, 2003; Zanchet et al., 2003).

Although the venoms of spiders of the genus *Phoneutria* do not appear to cause necrosis, this is a common pathogenic effect of other spider bites world wide, with the genus *Loxosceles* (recluse spiders) being particularly implicated. There have been suggestions that proteolytic enzymes in the venoms might be the causative agents of the dermonecrosis (Atkinson and Wright, 1992; Feitosa et al., 1998; Young and Pincus, 2001), but Foradori et al. (2001) have shown that their role is most probably a secondary one. The venom component which most actively participates in the formation of skin lesions appears to be sphingomyelinase D (Futrell, 1992), and there have been comparative studies of the phylogenetic distribution of this enzyme in the venoms of a wide range of spiders (Young and Pincus, 2001; Binford and Wells, 2003; Foradori et al., 2005; Machado et al., 2005). So far we have been unable to detect this enzyme in any of the *Phoneutria* venoms.

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PN47 IVYGTVTTPGKYPWMVSIHERVKDVMKQ-ACGGAILNENWIVTAAHCFDQ
PN44 IVGGKPLSGGLQPWMVTLH-VKYDKEFEHVCGGSILNERWIFTAAH....
AGPI IVGGKTAKFGDYPWMVSIQQKNKKGTFDHICGGAIINVNWILTAAHCFDQ
BSS  IVGGTTVTHGSIPWQVSLRLKRELR---HFCGGSILNRNWILTAAHCIRK

PN47 PIILKDYEVYVGIVSWLHKNAPTVQKFQLSKIIIHDKYVKDGFANDIALI
AGPI PIVKSDYRAYVGLRSILHKKENTVQRLELSKIVLHPGYKPKKDPDDIALI
BSS  PQQPKKYLAILGDYDRIQYDFSEMKVGFRL-IFNHEKYNPATFENDITLM

PN47 KTATPIDIKGSKGYVNGICFPSGATDPSGEATVIGWGMIRGGGPISAELR
AGPI KVAKPIVIGN---YANGICVPKGVTNPEGNATVIGWGKISSGGKQVNTLQ
BSS  KMDTSISIATIFGQSVFPPANKVPAAKSKII-VSGWGDTKGTTQDVK-LN

PN47 QVTLPLVPWQKCKQIYGHPDSEFEYIQVVPSMLCAGGN--GKDACQFDSG
AGPI EVTIPIIPWKKCKEIYGDEFSEFEYSQITPYMICAGAE--GKDSCQADSG
BSS  QVTLPVMSKKLCKKLYSKVVGAAPVFKTS---LCAAYKKGGKDSCQGDSG

PN47 GPLFQYDKKGVATLIGTVANGADCAYAHYPGMYMKVSAFRSWMDKVMT
AGPI GPLFQIDANGVATLIGTVANGADCGYKHYPGVYMKVSSYTNWMSKNMV
BSS  GPLVQKSKSGNWQVVGIVSWGVGCALERKPSVNTMVSKYIDWIENKMNNF
```

Fig. 4. Comparison of the amino acid sequences of the serine proteases PN47 and PN44 from the venom of the spider *Phoneutria nigriventer* with the peptide isomerase from the venom of the spider *Agelenopsis aperta* (AGPI) (Shikata et al., 1995), and a protease from the brown sea squirt *Herdmania momus* (BSS) (Arnold et al., 1997). (–) represent gaps inserted to facilitate comparison of the sequences.

During our most recent studies we have determined the complete or partial amino acid sequences of more than 100 peptides/proteins purified from the venoms of the three species of *Phoneutria* spiders. We now present the structures of some 60 of these molecules, but have omitted the details of those which were found to be only minor isoforms of previously described components of the venom of *P. nigriventer*.

We have found that the venoms of the spiders *P. keyserlingi* and *P. reidyi*, as might be expected, contain a series of polypeptides which are very similar, but not identical, in their amino acid sequences and biological activities, to the proteins previously described by us from the venom of *P. nigriventer*. A comparison of the alignment of the amino acid sequences of these *Phoneutria* proteins is most easily made when they are divided into two groups, on the basis of whether they contain the sequence motifs CxCC (Fig. 5) or CC (Fig. 6). It should be noted that, with few exceptions, the alignments shown in Figs. 5 and 6 strongly support the novel strategy recently suggested by Kozlov et al. (2005) for the identification of toxin-like structures amongst the components of spider venoms. After analysing about 150 polypeptides, these workers were able to identify a Principle Structural Motif (PSM), which postulates the existence of 6 amino acid residues between the first and second cysteine residues, and a Cys–Cys sequence at a distance of 5–10 amino acid residues from the second cysteine; and also an Extra Structural Motif (ESM), which postulates the existence of a pair of CxC sequences in the C-terminal region. Inspection of the sequences in Fig. 5 reveals that all of the *Phoneutria* proteins shown obey these two motif rules. On the otherhand in Fig. 6 there are some exceptions, notably the absence of the ESM (two CxC sequences in the C-terminal regions) in the smaller (<4kDa.) toxins from *Phoneutria*.

Fig. 5a shows the structures of PK15C1, PK16C1 and PR14C1, all of which have almost identical amino acid sequences with that of the previously reported PNTx1 (Diniz et al., 1990). All four toxins induce the same excitatory symptoms (tail erection, agitation, lachrymation, hypersalivation, spastic paralysis) and rapid death within 5–8 min, following injection (icv) in mice at dose levels of 1.5 μg/mouse. Preliminary studies on the binding of [^{125}I]-PNTx1 suggest that this toxin acts on Ca^{2+} ion channels, which are probably of the L-type (Santos et al., 1999).

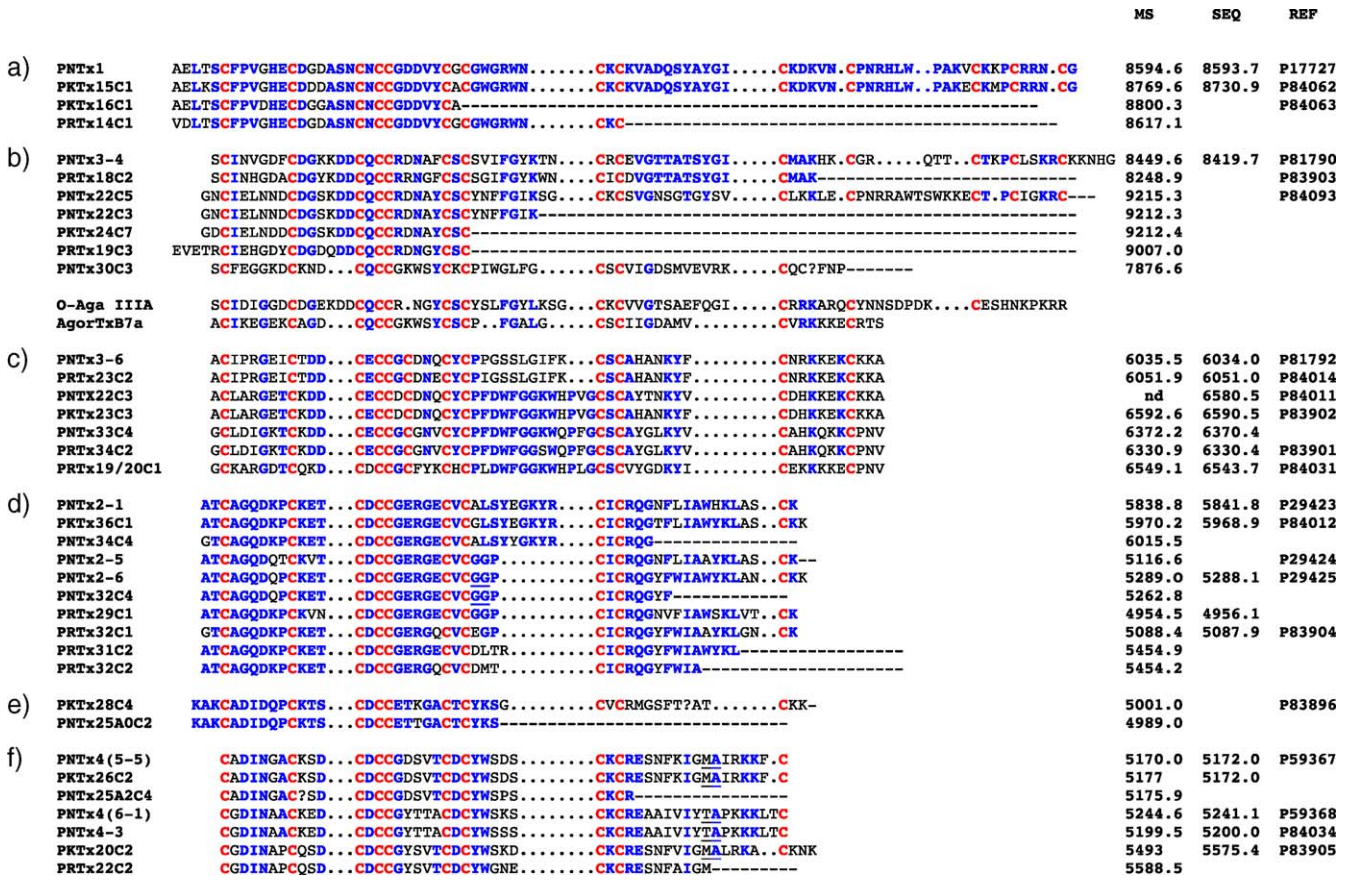

Fig. 5. Alignments of the amino acid sequences of toxic peptides purified from the venoms of the Brazilian spiders *Phoneutria nigriventer* (PN), *Phoneutria reidyi* (PR), and *Phoneutria keyserlingi* (PK), which contain the sequence motif CxCC. (..), gaps introduced to facilitate the alignment of the Cys (C) residues; (–), C-terminal sequences not yet determined; (?), amino acid not identified; MS, molecular masses (Da) determined by mass spectroscopy (MALDI-TOF or Q-TOF); SEQ, molecular masses calculated from complete amino acid sequences; REF, shows Accession number of sequences deposited in SWISS-PROT/TREMBL data base. The underlinings of parts of some sequences indicate those peptide bonds which were shown to suffer low levels of proteolytic nicking or hydrolysis. The sequences are arranged in groups to show the families which have strong sequence identities with (a) PNTx1, (b) PNTx3-4, (c) PNTx3-6, (d) PNTx2, (e) new family, and (f) PNTx4. Also shown for comparison are the sequences of toxins from other spiders; o-Aga IIIA, omega-Agatoxin IIIA from *Agelenopsis aperta* (Venema et al., 1992) and AgorTxB7a from *Agelena orientalis* (Kozlov et al., submitted for publication).

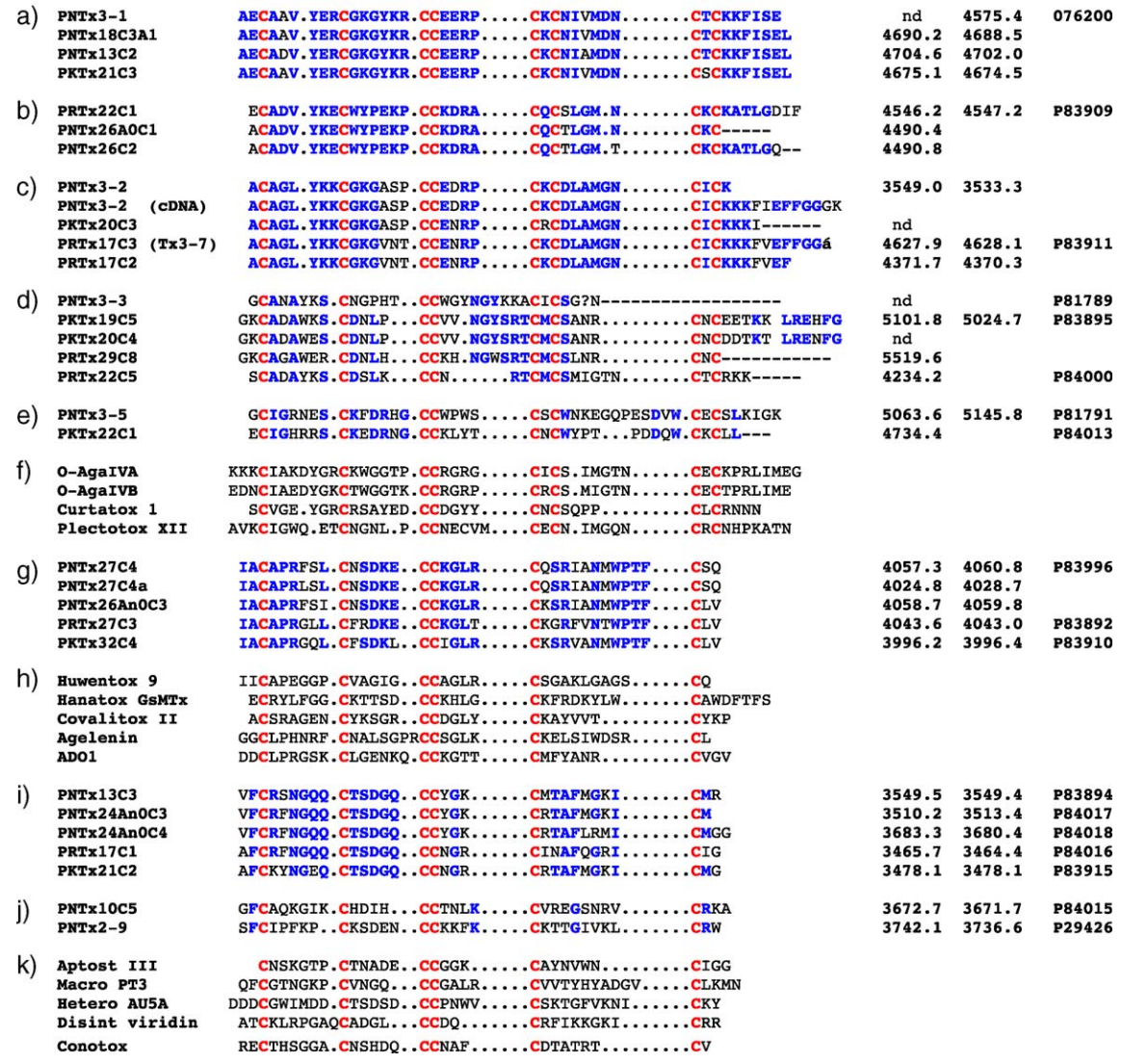

Fig. 6. Alignments of the amino acid sequences of toxic peptides purified from the venoms of the Brazilian spiders *Phoneutria nigriventer* (PN), *Phoneutria reidyi* (PR), and *Phoneutria keyserlingi* (PK), which contain the sequence motif CC. #, signifies that the C-terminal G was amidated. The groups/families shown are (a) PNTx3-1; (b) new family; (c) PNTx3-2; (d) PNTx3-3; (e) PNTx3-5; (g) new 4 kDa family; (i) new 3.5 kDa family of insecticidal peptides; (j) other *Phoneutria* proteins. Also shown for comparison in f are the omega-Agatoxins IVA and IVB (Mintz et al., 1992; Adams et al., 1993), curtatoxin1 (Stapleton et al., 1990) and plectotoxin XII (Quistad and Skinner, 1994); in panel h huwentoxin-9 from the Chinese bird spider (*Selenocosmia huwena*) (Liang, 2004), peptide GsMTx2 from the Chilean rose tarantula (*Grammostola spatulata*) (Oswald et al., 2002), covalitoxin II from the Singapore tarantula (*Coremiocnemis validus*) (Balaji et al., 2000), agelenin from *Agelena opulenta* (Hagiwara et al., 1991), and ADO1, a Ca^{2+} channel toxin from the assassin bug *Agriosphodrus dohmi* (Corzo et al., 2001); and panel k shows small insecticidal peptides from other spiders such as Apto III from the trap-door spider (*Aptostichus schlingeri*) (Skinner et al., 1992), peptide toxin 3 (PT3) from *Macrothele gigas* (Satake et al., 2003), and the Heteropodatoxin AU5A from the giant crab spider *Heteropoda venatoria* (Kelbaugh et al., 1997), the disintegrin viridin (an inhibitor of the activation of platelet aggregation) from the venom of the rattle-snake *Crotalus viridis* (Scarborough et al., 1993) and also a conotoxin from the predatory cone snail *Conus* (Duda and Palumbi, 2000) (k). cDNA, amino acid sequence deduced from cDNA sequence (Kalapothakis et al., 1998a). Other details are the same as given for Fig. 5.

Also shown in Fig. 5b are the amino acid sequences of a number of new toxins purified from the three *Phoneutria* venoms which have strong similarities in their structures and biological activities with the PNTx3-4 previously described only from *P. nigriventer* (Cordeiro et al., 1993; Cassola et al., 1998). This type of toxin when injected in mice initially caused paralysis of the posterior limbs followed by general flaccid paralysis and eventually death after 8–10 min. Electrophysiological studies have indicated that TX3-4 toxin blocks Ca^{2+} channels of the N-type and P/Q-type (Cassola et al., 1998; Santos et al., 2002; Troncone et al., 2003). Both the Tx1 and Tx3-4 types of neurotoxins from the *Phoneutria* spiders contain 14 cysteine residues (probably seven disulfide bridges), and have approximately 40% sequence homology with one another and with the omega-Agatoxins III from the venom of the spider *A. aperta*, which are antagonists of N- and L-type Ca^{2+} channels (Venema et al., 1992), but the latter only contain 12 cysteines.

Also shown in Fig. 5c are the structures of the Tx3-6 family of toxins found in the *Phoneutria* venoms, which exhibit high (60–96%) sequence identities with one another, some similarities with the *Phoneutria* Tx3-4 group, and 40–50% homology

with the omega–AgatoxinsIII and the Agor Tx B7a toxin from the venom of the spider *Agelena orientalis* (Kozlov et al., submitted for publication). This *Phoneutria* Tx3-6 family which like the omega–AgatoxinsIII contains 12 cysteines, is distinguished by having the sequence motif CxCCxC which is unique to the family. The toxins of this type from *Phoneutria* are of relatively low toxicity when injected in mice. Dose levels of 5 μg/mouse produced paralysis only in the posterior limbs and gradual decreases in movement and aggression during a 24 h period (Cordeiro et al., 1993). Vieira et al. (2003) have shown that PNTx 3–6 inhibits K^+-evoked increases in $[Ca^{2+}]_i$ and Ca^{2+}-dependent glutamate release in rat synaptosomes, and that this inhibition involves P/Q-type calcium channels. Another recent study by our collaborators has investigated the effects of purified PNTx3-6 on cloned mammalian Ca (2+) channels expressed in human embryonic kidney 293 cells, and endogenous Ca(2+) channels in N18 neuroblastoma cells, using whole-cell patch-clamp measurements (Vieira et al., 2005). These experiments confirmed that PNTx3-6 acts as an omega-toxin that targets high voltage-activated Ca(2+) channels, with a preference for the Ca(v)2 family of N-, P/Q-, and R-types.

The two most toxic peptides in the venom of *P. nigriventer* are Tx2-6 and Tx2-5, both of which cause death within 2–5 min when injected icv in mice at dose levels of 0.5 μg/mouse (Cordeiro et al., 1992; Araujo et al., 1993a,b). We have previously shown that the PNTx2-5 and PNTx2-6 type of toxins prolong the inactivation and deactivation of sodium ion channels (Araujo et al., 1993a,b; Matavel et al., 2002). We have also observed that when Tx2-6 was injected subcutaneously at 1.5 μg/mouse it caused penile erection (priapism) within 25–30 min, followed by death after 50–80 min (Richardson and Cordeiro, unpublished results). Similar observations have been reported by Troncone et al. (1998) for PNTx2-6, and recently Yonamine et al. (2004) have shown that Tx2-5 also causes priapism when injected intraperitoneally at a dose level of 10 μg/mouse. Furthermore these workers demonstrated that pretreatment of the mice with 7-nitroindazole, a selective inhibitor of neuronal nitric oxide synthase, prevented the toxic effects of Tx2-5. Other research on the sodium channel toxins from scorpion venoms, which similarly induce priapism and death, has also implicated the activation of nitric oxide synthases in the development of the toxic syndrome (Fernandes et al., 2003; Teixeira et al., 2003). As can be seen in Fig. 5d the venoms of *P. reidyi* and *P. keyserlingi* both contain toxins with amino acid sequences that show high (73–94%) sequence identities with the PNTx2 type of lethal neurotoxins acting on Na^+ channels. These proteins from *P. reidyi* and *P. keyserlingi* elicited very similar toxic and lethal effects to the *P. nigriventer* isoforms when tested in mice.

It is notable that the Tx2 family of peptides investigated during this study quite frequently exhibited low levels of proteolytic "nicking" or cleavage of internal peptide bonds. For example when the mass of the toxin PNTx32C4 (an isoform of Tx2-6) was determined by Q-TOF mass spectroscopy it yielded a strong signal at 5262.8 Da and a weaker signal at 5280.7 Da, the difference of 18 Da being the result of the cleavage of a peptide bond. The location of the cleaved peptide bond was subsequently identified when the native peptide was subjected to automated sequencing and two amino acid sequences were obtained. The strongest sequence was ATxAGQDQP, which is the N-terminal of the protein, but the weaker sequence was GPxIxRQGY, which clearly resulted from the hydrolysis of the peptide bond $G_{26}–G_{27}$ (Fig. 5d).

We have recently isolated two new peptides (PKTx28C4 and PNTx25A0C2), both of which are very toxic for both mice and house-flies. As can be seen in Fig. 5e they have 47–51% sequence homology with theTx2 family, and 43–47% identity with the insecticidal Tx4 toxins (shown in Fig. 5f).

We have previously described another family of peptides from the venom of *P. nigriventer* which produced no obvious toxic effects in bioassays with mice, but were extremely lethal to house-flies and a number of other insects (Figueiredo et al., 1995, 2001; Oliveira et al., 2003). This group of toxins (PNTx4) seems to act at the level of the peripheral nervous system in insects stimulating glutamate release at neuromuscular junctions. Despite not causing macroscopic effects in mice, these proteins inhibit glutamate uptake in rat brain synaptosomes (Mafra et al., 1999), and one of them PNTx4(5-5) was shown to selectively inhibit the NMDA-subtype of ionotropic glutamate receptor in rat brain neurons (Figueiredo et al., 2001). Fig. 5f shows that the venoms of both PK and PR also contain peptides of this type. We have also detected the presence of low levels of "nicked" forms of these molecules in which the peptide bonds $M_{40}–A_{41}$ in PKTx26C2, PRTx22C2 and PNTx4(5-5), and $T_{40}–A_{41}$ in PNTx4(6-1) and PNTx4(6-3) were found to have been hydrolysed.

The first group of toxins shown in Fig. 6a which have the sequence motif CC instead of CxCC are the PNTx3-1 family of isoforms, which so far have only been isolated from *P. nigriventer* and *P. keyserlingi*. These peptides caused paralysis only in the hindlimbs and gradual decreases in movement and aggression when tested in mice. Kushmerick et al. (1999) have used whole-cell patch clamp experiments to demonstrate that PNTx3-1 blocks A-type K^+ currents controlling Ca^{2+} oscillation frequencies in GH_3 cells. A recombinant mutant form of PNTx3-1 which has the extra sequence ISEF as an extension at the N-terminus has been produced in a bacterial expression system, and was found to block A-type K(+) currents in GH_3 cells in the same manner as the wild-type protein (Carneiro et al., 2003).

The three peptides shown in Fig. 6b have very similar (>90% identity) amino acid sequences to the predicted structure of the neurotoxin PN3A obtained from cDNA sequencing (Kalapothakis et al., 1998a,b), but this group is also clearly related to the toxins of the Tx3-1 (Fig. 6a) and Tx3-2 (Fig. 6c) types and has 40–44% identity with the omega-Agatoxins IVA and IVB from the spider *A. aperta*, which are antagonists of P-type calcium channels (Mintz et al., 1992; Adams et al., 1993).

Toxins of the PNTx3-2 type have now been found in all three species of *Phoneutria* (Fig. 6c). When injected in mice (icv) these toxins induce immediate clockwise gyration and flaccid paralysis after 5–6 h. Electrophysiological studies by Kalapothakis et al. (1998b) have shown that PNTx3-2 caused a

progressive decrease in current in L-type calcium channels in GH_3 cells. It is noteworthy that toxins of this type particularly appear to undergo post-translational processing in the C-terminal regions of their polypeptide chains. The first example of the toxin PNTx3-2 reported by us contained only 34 amino acids and had a mass of 3549.0 Da (Cordeiro et al., 1993), but subsequently Kalapothakis et al. (1998a) reported that the results of sequencing of cDNA indicated that the nascent protein might have an additional 10 or 11 amino acids at the C-terminus. Although we have not so far succeeded in isolating examples of this longer form of Tx3-2 from *P. nigriventer*, we have purified two isoforms PRTx17C3 and PRTx17C2 from *P. reidyi* with respective masses of 4627.9 Da and 4371.7 Da. Inspection of the C-terminal sequences of these two proteins (Fig. 6c) strongly suggests that the former gave rise to the latter as the result of carboxypeptidase activity. However we have also demonstrated by using collision induced dissociation MS/MS analysis of the C-terminal peptide (FFGG) released from the native protein by digestion with a GLU-C specific proteinase that the C-terminal G_{43} residue of PRTx17C3 is amidated (Pimenta et al., unpublished results). It has been hypothesized that the key role of α-amidation is to prevent the ionization of the COOH-terminus of the peptide, rendering it more hydrophobic and, therefore, increasing its ability to bind to receptors (Betty et al., 1993). Furthermore it is thought that C-terminal amidation may act as a protective shield, preventing the cleavage of peptides by carboxypeptidases and thus increasing the half-life of small toxins inside the victim's tissues. In the case of the toxins PRTx17C3 and PRTx17C2 it is not known whether the C-terminal shortening occurred before or after the α-amidation event took place.

Fig. 6d shows the structures of the neurotoxins of the Tx3-3 type which have been purified from the *Phoneutria* venoms. These molecules have 42–89% sequence identity with one another, but also show some similarities (approximately 40%) to the *Phoneutria* Tx3-1 and Tx3-2 peptides and the omega-Agatoxins IVA and IVB (Mintz et al., 1992; Adams et al., 1993), plectotoxin XII (Quistad and Skinner, 1994), and curtatoxin1 (Stapleton et al., 1990) from other spider venoms (Fig. 6f). When injected in mice at dose levels of 5 μg/mouse these proteins caused rapid general flaccid paralysis and death in 10–30 min. Various workers have shown that PNTx3-3 is very potent in inhibiting calcium channels that regulate intracellular calcium changes (Prado et al., 1996; Guatimosim et al., 1997; Miranda et al., 1998) and was most effective in blocking P/Q- and R-type currents (Leão et al., 2000; Miranda et al., 2001).

Although we have isolated various C-terminally shortened isoforms of the toxin PNTx3-5 previously reported by us (Cordeiro et al., 1993), and have purified similar toxins from *P. keyserlingi* (Fig. 6e), we have been unable to find examples of peptides of this type in the venom of *P. reidyi*. The toxin PNTx3-5 was only mildly toxic when injected in mice, but like most of the Tx3 toxins appears to affect calcium channels (Beirao et al., unpublished results).

A new family of small (4 kDa) neurotoxic peptides discovered in all three species of *Phoneutria* during this study is shown in Fig. 6g. These molecules caused spastic paralysis and death when injected (icv) into mice at dose levels of 3.0 μg/mouse. Although three different isoforms were isolated from the venom of *P. nigriventer*, so far only single molecules have been found in the venoms of *P. keyserlingi* and *P. reidyi*. The peptides purified from the *Phoneutria* venoms exhibited between 70% and 95% sequence identity with one another, but also showed lower (50%) identity with the neurotoxin PNTx2-9 previously reported by us (Cordeiro et al., 1992). As well as having 34–52% levels of sequence homology with toxins from other spiders such as Huwentoxin-9 from the Chinese bird spider (*Selenocosmia huwena*) (Liang, 2004), peptide GsMTx2 from the Chilean rose tarantula (*Grammostola spatulata*) (Oswald et al., 2002), covalitoxin II from the Singapore tarantula (*Coremiocnemis validus*) (Balaji et al., 2000), and agelenin from *Agelena opulenta* (Hagiwara et al., 1991), this new family also had 52% sequence similarity with ADO1, a Ca^{2+} channel toxin from the assassin bug *Agriosphodrus dohmi* (Corzo et al., 2001) (Fig. 6h).

Fig. 6i shows the amino acid sequences of another new family of small peptides (3.5 kDa) found in the venoms of all three species of the genus *Phoneutria* which were examined during this study. These peptides appeared to have no observable toxic effects when they were injected (icv) into mice, but were very toxic and lethal when injected into house-flies. Other workers have reported small insecticidal peptides from other spiders such as the trap-door spider (*Aptostichus schlingeri*) (Skinner et al., 1992), *Macrothele gigas* (Satake et al., 2003), and the giant crab spider *Heteropoda venatoria* (Kelbaugh et al., 1997) which exhibit approximately 40% sequence identity with the members of this new family. Interestingly these small polypeptides also have some homology with a number of disintegrins (inhibitors of the activation of platelet aggregation) from the venoms of various snakes (Scarborough et al., 1993) and also with the conotoxins from the predatory cone snails (Duda and Palumbi, 2000) (Fig. 6k).

We anticipated that some of the toxins from the *Phoneutria* spiders reported here might exhibit greater sequence identities when compared with the toxins isolated from *Cupiennius salei* (Kuhn-Nentwig et al., 1994, 2000, 2004; Nentwig et al., 1998; Schaller et al., 2001; Wullschleger et al., 2004) as this other neotropical wandering spider also belongs to the family Ctenidae. In fact this was not the case, as the highest levels of sequence homology discovered between the *Phoneutria* and *Cupiennius* toxic peptides were only in the range of 33–35%, which is somewhat lower than the 40–52% values found in comparisons of the *Phoneutria* toxins with peptides from other spider families such as the Agelenidae (Figs. 5 and 6). This finding and the wealth of new sequence information for the toxins from *Phoneutria* venoms presented in this work in Figs. 5 and 6 add credence to the recent suggestion by Sollod et al. (2005) that the toxins from spider venoms "are produced in the form of structurally constrained combinatorial peptide libraries in which there is hypermutation of essentially all residues in the mature-toxin sequence with the exception of a handful of strictly conserved cysteines that direct the three-dimensional fold of the toxin".

We have found various peptides in these venoms of the genus *Phoneutria* which do not cause any obvious toxic effects when they are injected into mice or house-flies. One of these is PNTx22A0C1, a 4 kDa polypeptide which has approximately 50% sequence similarity with PNTx2-9, a previously reported Na^+ channel toxin from *P. nigriventer* (Cordeiro et al., 1992), and lower homologies (30–36%) with the small *Phoneutria* 3.5 kDa and 4 kDa toxins reported here, and with the AU5A and heteropodatoxin 1 from the giant crab spider (*H. venatoria*) (Kelbaugh et al., 1997; Sanguinetti et al., 1997), and the hannatoxin 2 from the Chilean rose tarantula (Swartz and MacKinnon, 1995), all of which block K^+ channels (Fig. 7). On the otherhand, as can be seen in Fig. 7 this peptide PNTx22A0C1 from spider venom has a more striking similarity in its N-terminal amino acid sequence with various proteinase inhibitors from the seeds of plants of the Cucurbitaceae, such as the bitter gourd (*Momordica charantia*) (Hayashi et al., 1994) and the cucumber trypsin inhibitor IV (Wieczorek et al., 1985). The spider peptide even has a recognisable reactive (inhibitory) peptide bond Lys_5–Ile_6 in the homologous position in its sequence (Fig. 7). We are currently investigating the possibility that PNTx22A0C1 may have activity as an inhibitor of serine proteinases such as trypsin.

Other larger apparently non-toxic peptides PNTx16C1, PRTx16C0 and PKTx18C1, purified from the three different *Phoneutria* venoms were found to have very similar (80% identity) amino acid sequences (Fig. 8). It is notable that two of the three *Phoneutria* venom proteins in this family have their N-termini blocked by the presence of a pyroglutamate residue. This type of post-translational modification is rare in the venom polypeptides from *Phoneutria*, and has only been previously reported for the small (0.9–1.4 kDa) phonetachykinins (Pimenta et al., 2005). These peptides whose masses are between 6981 and 7714 Da show low levels (32–37%) of sequence identity with proteins present in the dermal venoms of fire-bellied toads such as *Bombina maxima* (Chen et al., 2003) and *Bombina orientalis* (Chen et al., 2005) and the yellow-bellied toad *Bombina variegata* (Molloy et al., 1999), which potently contract gastro-intestinal smooth muscle and induce hyperalgesia (Fig. 8). The venom of the funnel web spider *Hadronyche versuta* also contains a polypeptide (atracotoxin-Hvf17) which is non-toxic to insects or mammals, but has sequence homology with this same group of venom proteins from *Phoneutria*, the *Bombina* toads, and an intestinal toxin in the venom of the mamba snake (Szeto et al., 2000; Wen et al., 2005). Recent pharmacological studies on atracotoxin-Hvf17 have shown that unlike the unlike the Bombina and mamba toxins, this peptide did not stimulate smooth muscle contractility, nor did it inhibit contractions induced by human PK1, and it failed to activate or block human PK1 or PK2 receptors (Wen et al., 2005). The presence

a) **Comparison with other spider toxins**

```
PNTx22A0C1  MPCPKILKQCKSDE-DCCRGWKCFGFSIKDKMCISR
PNTx2-9     SFCI-PFKPCKSDE-NCCKKF-CKKTTGIVKLCRW
PKTx21C2    AFCKYNGEQCTSDG-QCCNGR-CR-TAFMGKICMG
AU5A        DDCGWIMDDCTSD-SDCCPNWVCSKTGFVKNICKYEM
HeteropodTx1 DCGTIWHYCGTDQSECCEGWKCSRQ-----LCKYVIDW
HaTx2        ECRYLFGGCKTTA-DCCKHLGCK--FFRDKYCAWDFTFS
```

b) **Comparison with Cucurbit Trypsin inhibitors**

```
PNTx22A0C1  MPCPKILKQCKSDEDCCRGWKCFGFSIKDKMCISR
Momordica   RGCPRILKQCKQDSDC-PGE-CICMAHGF--CG
CUCUMBE Ti  MMCPRILMKCKHDSDCLPG--CVCLEHIEY-CG
                 |
            Inhibitory (Reactive) Site
```

Fig. 7. Comparison of the amino acid sequence of PNTx22A0C1, a peptide of unknown function from the venom of *Phoneutria nigriventer*, with toxins from other spider venoms (a), and with proteinase inhibitors from seeds of the plant family Cucurbitaceae (b). The other sequences shown in panel a are; PNTx2-9, a previously reported Na^+ channel toxin from *P. nigriventer* (Cordeiro et al., 1992), PKTx21C2 one of the small *Phoneutria* 3.5 kDa toxins reported here, the AU5A and heteropodatoxin 1 from the giant crab spider (*Heteropoda venatoria*) (Kelbaugh et al., 1997; Sanguinetti et al., 1997), and the hanatoxin 2 (HaTx2) from the Chilean rose tarantula (Swartz and MacKinnon, 1995); and in panel b, the bitter gourd (*Momordica charantia*) trypsin inhibitor (Hayashi et al., 1994), and the cucumber trypsin inhibitor IV (Wieczorek et al., 1985). The vertical line indicates the reactive (inhibitory site) peptide bond in the plant protein inhibitors of trypsin.

```
PNTx16C1 <QWIPGQSC-TNADCGEGQCCTGGSYNRH---CQSLSDDGKPC
PRTx16C0       EWCGSNADCGDGQCCTGGSFNRH---CQSLADDGTPC
PKTx18C1 (.................)CCTGGSYNRH---CQSLSDDGKPC
Bomva 8   AVITGA-CDKDVQCGSGTCCAASAWSRNIRFCTPLGNSGEDC
Bomor     AVITGA-CDRDVQCGSGTCCAASAWSRNIRFCVPLGNSGEEC
Bommx     AVITGV-CDRDAQCGSGTCCAASAFSRNIRFCVPLGNNGEEC
ATRACOT         EQCGDD-VCGAGHCCSE--YPMH---CKRVGQLYDLC
CAERON     CDAYGDCKKNSDCKAGECCVNTPPFARST-CQKYLQQGEFC

PNTx16C1  QRPNKYDEYKFG-----CPCKEGLMCQVINY---CQK  (7666.6)
PRTx16C0  QKPNDYNEYKFG-----CPCKQGLICSPINY---CQKK (6981.3)
PKTx18C1  QRPNKYDEYKFG-----CPCKEGLLCSVINY---CQK  (7714.1)
Bomva 8   HPASHKVPYDGKRLSSLCPCKSGLTCSKSGEKFKCS
Bomor     HPASHKVRYDGKRLSSLCPCKSGLTCSKSGAKFQCS
Bommx     HPASHKVPSDGKRLSSLCPCNTGLTCSKSGEKYQCS
ATRACOT   MASKATKNSGNHLFF--CPCDEGMYCDMNSWS--CQKRT
CAERON    AHMGKYNPLGKYINM--CPCGKGLKCQLK
```

Fig. 8. Comparison of the amino acid sequences of the non-toxic PNTx16C1, PRTx16C0, and PKTx18C1 type of venom peptides from *Phoneutria* spiders with peptides isolated from the dermal venoms of the yellow-bellied toad *Bombina variegata* (Bomva 8) (Molloy et al., 1999), and the fire-bellied toads *Bombina orientalis* (Bomor) (Chen et al., 2005) and *Bombina maxima* (Bommx) (Chen et al., 2003), the atracotoxin from the funnel web spider *Hadronyche versuta* (Szeto et al., 2000), and the putative toxin caeron from the bark spider *Caerostris extrusa* (Daí et al., 2001). The molecular masses of the *Phoneutria* proteins are shown in brackets at the end of each sequence. <Q, indicates N-terminal is blocked by pyroglutamate residue; (.....) part of sequence not determined.

```
PN16C3  ARPKSDCEKHRESTEKTGTIMKLI---------PKCKENSDYE
Equist  IRGSPDCSRRKAALTLCQMMQAIIVNVPGWCGPPSCKADGSFD
OPI-2   IRPKTPCEDARDATLN-GSIGAYI---------PTCDDSGQYT

PN16C3  ELQCYEDSKFCVCYDKKGHAASPISTKVKE-CGCYLKQKERK-
Equist  EVQCCASNGECYCVDKKGKELEGTRQKGRPSCERHLSPCEEAR
OPI-2   PEQCWGS-GYCWCVTSTGQKIPGTETPPGTAPINCGSTQNGMI

PN16C3  ------DSGRESAII--------PQCEEDGKWAKKQLWEFNKS
Equist  LQAHS-NSLRVGMFV--------PQCLEDGSYNPVQCWPSTGY
OPI-2   RPKTPCEIARDTAMKKVRPGVYIPTCDNDGRYTPEQCSGSTGY

PN16C3  CWCVDEKGEQVGKIHHDCDSLK-CE
Equist  CWCVDEGGVKVPGSDVRFKRPT-C
OPI-2   CWCVTSSGQKIQGTETPPGTAINCST
```

Fig. 9. Comparison of the amino acid sequence of the non-toxic 14.7 kDa protein PN16C3 from the venom of *Phoneutria nigriventer* with the sequences of equistatin from the European sea anemone *Actinia equina* (Lenarcic et al., 1997), and the protein OPI-2 from trout oocytes (Wood et al., 2004), both of which are inhibitors of cysteine proteinases.

of a similar putative toxin in the venom of the bark spider *Caerostris extrusa* has been indicated by the sequencing of cDNA (Daí et al., 2001) (Fig. 8).

The complete amino acid sequence of another much larger (14770 Da) non-toxic protein PN16C3 purified so far only from the venom of *P. nigriventer* shows no sequence homology with any other protein from the venoms of *Phoneutria* or other spiders, but has 30% sequence identity with equistatin isolated from the European sea anemone (*Actinia equina*), which is an inhibitor of cysteine proteinases (Lenarcic et al., 1997) (Fig. 9). The sequence of the latter protein appears to contain three thyroglobulin (type 1)-like domains, whereas only two are apparent in the smaller molecule of PN16C3. There is also 27–29% sequence similarity with two cysteine proteinase inhibitors from trout oocytes (Bobe and Goetz, 2001; Wood et al., 2004). We are currently investigating the possibility that PN16C3 is an inhibitor of thiol proteinases.

It should be noted that there is some controversy over the exact taxonomic position of *P. keyserlingi*. Eickstedt (1979) distinguished *P. nigriventer* from *P. keyserlingi* by the morphological characteristics of larger epigynal lateral guides and less curved embolus of the latter species. However Simo and Brescovit (2001) consider that these differences only represent intraspecific variability, and suggest that *P. keyserlingi* should be regarded as a synonym of *P. nigriventer*. The results of this present biochemical investigation in which the amino acid sequences of 15 proteins isolated from the venom of *P. keyserlingi* can be compared with those from *P. nigriventer* show that whilst there are strong similarities between these molecules, there are also substantial differences. Hopefully when we are able to compare the sequences of a larger number of the peptides/proteins from the venoms of *P. nigriventer* and *P. keyserlingi* these molecular data may help in resolving this taxonomic problem.

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