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Mass spectrometry analysis, amino acid sequence and biological activity of venom components from the Brazilian scorpion *Opisthacanthus cayaporum* ☆

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ABSTRACT

This communication reports the separation of 80 fractions from the venom of the Ischnuridae scorpion *Opisthacanthus cayaporum* by high-performance liquid chromatography (HPLC). From these, 93 distinct components were identified by liquid chromatography/electrospray mass spectrometry (LC/ESI-MS) analysis, with molecular weights varying from 229.2 to 61,144.0 atomic mass units. Additionally, the HPLC fractions were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) which resulted in 221 distinct components, among which were 52 of the 93 obtained by LC/ESI-MS. The entire set of different molecular species found (total of 262 molecular masses) has a trimodal molecular weight distribution, with 42% of the components possessing 229.2–2985.3 Da, 37% within the range of 3045.0–7258.6 Da and 12% within the range 7458.4–9429 Da. Seventeen peptides/proteins were isolated and were sequenced by Edman degradation, among which were a scorpine-like peptide (8315 Da), presenting antimicrobial activity, and two phospholipase A2 with a molecular weight around 14 kDa. The pharmacological effects of the venom were tested on isolated rat and insect (cockroach) nerves using the single sucrose-gap assay. The ED50 of the venom was 1.1 mg/ml in insect nerves. Venom concentrations in the order of 3 mg/ml causes only 9% reduction of compound action potentials (APs) of rat nerves, suggesting that this venom is rather specific for insects. Comparative analysis of venom from male and female *O. cayaporum* was performed by HPLC and MALDI-TOF-MS showing no qualitative variations, but rather quantitative differences among both samples.

1. Introduction

Many organisms produce and secrete venom for defense and/or capture of preys. These secretions are a rich source of pharmacologically active compounds such as enzymes, toxic peptides, low molecular weight substances and proteins of unknown functions. Toxins isolated from venoms affect with high selectivity and affinity a large number of targets, among which are ion channels, acetylcholine receptors, enzymes, cell

☆ *Ethical statement*: The scorpions are milked after CO2 anesthesia. No direct assays were performed with other animals alive. The physiological experiments were performed with tissues recovered from animals according to standard procedures approved by the ethical committee of our Institutions.

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membrane polarity and various hemostatic pathways. Toxic peptides isolated from venomous animals are usually of small sizes, ranging from 8 to 70 amino acids, which are highly compacted and stabilized by either disulfide bonds or by hydrogen bonding interactions. Some toxic peptides show post-translational-modified amino acids such as those from cone snails and amphibians (reviewed in Buczek et al., 2005; Auvynet et al., 2006, respectively).

Among the venomous animals are the scorpions. During about 400 millions years, they have successfully developed a large variety of bioactive peptides. These peptides contain 13–76 amino acids which can be classified into two types: disulfide-bridged or non-disulfide-bridged containing peptides. Scorpion toxins with disulfide bridges specifically interact with membrane-bound ionic channels including Na^+, K^+, Cl^- and Ca^{2+} channels (reviewed in Catterall et al., 2007; Possani et al., 1999). The Na^+-channel-specific toxins (Na^+-toxins) are composed of 58–76 amino acid residues linked by four disulfide bridges, whereas those specific for K^+ channels (K^+-toxins) and Cl^- channels are short-chain peptides composed of 20–41 amino acid residues and stabilized by three or four disulfide bridges (Possani and Rodríguez de la Vega, 2006). Recently, some "long-chain" K^+-channel toxins and Na^+-channel toxins that are constrained by only three disulfide bridges have also been reported (Diego-García et al., 2007). Toxins affecting Ca^{2+} channels possess a more variable amino acid length (Zhu et al., 2004a; Gurrola et al., 2005; Zhijian et al., 2006). Thus far, the best-known groups are those acting on Na^+ and K^+ channels. Currently, about 190 Na^+-channel toxins, 130 K^+-channel toxins, 17 Cl^--channel toxins and 5 Ca^{2+}-channel toxins have been described (Scorpion Toxin Database, see: http://sdmc.i2r.a-star.edu.sg/scorpion/). These ion channel blockers or modulators have been widely used as tools for the identification, isolation and characterization of ion channel proteins, including their pharmacological and physiological functions. Fewer non-disulfide-bridged components have been isolated and characterized from scorpion venoms, among which are peptides showing bradykinin-potentiating, antimicrobial, hemolytic and immune-modulating activities (Zeng et al., 2005). Proteomic approach was conducted with nine scorpion species documenting the overall composition of their venom gland secretion. All of these studies were done with scorpions from the family Buthidae and most of them belonging to the *Tityus* genus (Pimenta et al., 2001; Batista et al., 2004, 2006; Diego-García et al., 2005; Barona et al., 2006; Borges et al., 2006; Caliskan et al., 2006; Nascimento et al., 2006; Favreau et al., 2006).

Further insights into scorpion venom compositions were achieved by gene cloning using PCR-based methods conducted with cDNA libraries as template. Recently, the first transcriptome analysis of genes transcribed by the venomous gland of a scorpion was reported (Schwartz et al., 2007a).

Nothing or very little is known (a congress report, Schwartz et al., 2007b) about the venom from the Brazilian scorpion *Opisthacanthus cayaporum*, from the Ischnuridae family, which is the object of this communication. This family includes the genera *Opisthacanthus*, *Hadogenes* and *Cheloctonus* which are not venomous to man, and a sting from one of these genera should cause no more damage than that of a bee sting. Due to its lower toxicity, there have been few reports on the venom of scorpions from the Ischnuridae family. This family is distributed through Africa, South-East Asia, Australia and South America and associated islands. *Opisthacanthus* occurs in the Caribbean, Central and South Americas, Africa and Madagascar. The genus *Opisthacanthus* presents a gondwanian pattern of distribution. In Brazil the family Ischnuridae is represented by two species. *O. cayaporum* is endemic to open savannas in the eastern Amazonian (South of the State of Pará and State of Tocantins). From the venom of the species *Opisthacanthus madagascariensis*, endemic of Madagascar, cytotoxic linear peptides (IsCT and IsCT2, Dai et al., 2001, 2002) and K^+-channel blockers (IsTX and Om-toxins, Yamaji et al., 2004; Chagot et al., 2005) have been identified and studied.

In this communication we report the results of proteomic, biochemical and pharmacological characterizations of the soluble venom extracted from the scorpion *O. cayaporum*. The molecular masses of the venom components were identified after high-performance liquid chromatographic separation (HPLC) of the venom. Several of the main HPLC components were sequenced. Pharmacological assays were performed using both insects and rat nerve cells. Finally, a comparative MALDI-TOF-MS analysis was performed using samples from male and female *O. cayaporum* in order to verify the existence of possible sex-linked venom variations.

2. Material and methods

2.1. Venom source and purification procedures

Scorpions of the species *O. cayaporum* were captured in Palmas (State of Tocantins, Brazil) under the Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis (IBAMA) license number 048/2007-CGFAU. The crude venom from 15 adult scorpions of both sexes was obtained by electrical stimulation and dried. The material was dissolved in water and centrifuged at 10,000*g* for 10 min. The soluble supernatant was stored at −20 °C. When needed a sample was separated by HPLC in a C18 reverse-phase analytical column (Vydac, Hisperia, CA), using a linear gradient from 0% solvent A (0.12% trifluoroacetic acid, TFA, in water) to 60% solvent B (0.10% TFA in acetonitrile) run for 60 min, at a flow rate of 1 ml/min. In this conditions some peptides were obtained in homogeneous form; others required a second HPLC procedure, using an analytical C18 reverse-phase column, run with slightly modified gradients to improve separation, as described in the figure legends.

In order to compare the chromatographic profiles of male and female venoms, the venom of scorpions of each sex were separately obtained and submitted to HPLC fractioning as described above.

2.2. Mass spectrometry determination

Five microliters of each chromatographic fraction were injected into an LC/MS system composed of a Finnigan LCQDuo ion trap mass spectrometer (San Jose, CA) with a nanoelectrospray ionization (ESI) source using a Surveyor MS syringe pump delivery system and a C18 PicoFrit column/needle (75 mm × 10.1 cm) which was set on an *xyz* multi-axis translational stage for optimizing the ESI signal. The LC/ESI-MS/MS data acquisition program was set up to collect ion signals from the eluted peptides using an automatic, data-dependent scan procedure, in which a cyclic series of three different scan modes (full scan, zoom scan and MS/MS scan) were performed. First, the most abundant peptide ion peak in a full scan (*m/z* 300–2000) was selected as the precursor ion. Second, a zoom scan was performed to determine the charged state of the precursor ion. Finally, an MS/MS scan was used to determine the sequence of the precursor ion using collisionally induced dissociation with relative collision energy of 40%. The data acquisition and the automatic analysis of data were performed with the Xcalibur software on a Windows NT PC system.

All chromatographic fractions were also submitted to molecular mass determination by using an UltraFlex II MALDI-TOF/TOF Mass Spectrometer (Bruker Daltonics, Billerica, MA). Chromatographic fractions were dissolved in an α-cyano-4-hydroxycinnamic acid matrix solution (1:3, v:v), spotted onto a MALDI target plate and dried at room temperature for 15 min. The monoisotopic masses were obtained in linear and reflector modes with external calibration, using the Peptide Calibration Standard for Mass Spectrometry calibration mixture (up to 4000 Da mass range, Bruker Daltonics).

The determination of molecular masses of female and male venom compounds were performed by MALDI-TOF-MS. Venoms (1 and 10 μg quantities) were dissolved in an α-cyano-4-hydroxycinnamic acid matrix solution (1:3, v:v), spotted in triplicates onto a MALDI target plate and dried at room temperature for 15 min. The compounds monoisotopic (600–6000 Da) or average (4000–40,000 Da) masses were obtained in a positive reflector mode with external calibration, using the Peptide Calibration Standard for Mass Spectrometry calibration mixture (up to 4000 Da mass range, Bruker Daltonics).

2.3. Amino acid sequence determination

Homogeneity of peptides was verified by mass spectrometry and amino acid sequencing. Amino acid sequence determination of pure peptides was performed by automatic Edman degradation in a Beckman LF 3000 Protein Sequencer (Palo Alto, CA, USA) using the chemicals and procedures described previously (Schwartz et al., 2006). Similarly, searches were performed using BLAST (www.ncbi.nlm.nih.gov/blast) and FASTA 3 (www.ebi.ac.uk/fasta).

2.4. Antimicrobial activity

The protein content of the purified Ocy39.87 peptide was estimated by measuring the absorbance at 280 nm. It was assumed that one unit of absorbance in a 1 cm cell pathway is equal to 1 mg/ml concentration. The activity towards *Staphylococcus aureus* (ATCC 29213) was tested by the liquid growth inhibition test (Diego-García et al., 2008). Briefly, the peptide (15μg/ml final concentration) was added to a fresh bacterial inoculum grown in Luria Bertani (LB) sterile liquid medium with a cell density of approximately 1.5×10^5 colony-forming units/ml. Positive and negative controls were carried with the inoculum plus LB medium and LB medium only, respectively. The experiments were performed in triplicates. The spectrophotometric readings (596 nm) were performed after 12 h incubation time. The antibacterial activity of Ocy39.87 was calculated as the percentage of bacterial growth as compared to the positive control.

2.5. Phospholipase activity

Phospholipase A_2 activities of two components (Ocy 37.75 and Ocy39.10) were assayed by the clearing of a hen's egg yolk suspension incorporated into agarose gels (Habermann and Hardt, 1972).

2.6. Single sucrose gap assay and dose– response curve

The crude venom used in electrophysiological experiments was prepared in adequate buffers as follows. For rat, Krebs–Ringer, pH 7.2, solution contains in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11.1, HEPES 5.0. For cockroach the physiological solution was constituted in mM: NaCl 214, KCl 3.1, $CaCl_2$ 1.8, Na_2HPO_4 1.8 and NaH_2PO_4 0.2.

Compound action potentials (APs) of cockroach (*Periplaneta americana*) and rat (*Rattus norvegicus*) nerves were recorded with a single sucrose-gap technique, as described before (Wagner et al., 2003). The experiments using cockroach nerves were carried out at room temperature (24–28 °C), and the experiments with rat nerves were done at 31–33 °C. Recordings of the APs elicited by the stimulation were made in a Tektronix TDS 360 Oscilloscope (Tektronix Inc., USA) through a differential amplifier. The dose–response curve was obtained by a monolog function from RT-Plot Science v.2.8 (http://www.programsarchive.com/).

3. Results and discussion

3.1. Separation of the venom components, mass fingerprinting and partial N-terminal amino acid sequences

The separation procedure of *O. cayaporum* venom by HPLC resulted in the observation of at least 80 fractions (Fig. 1). By using ESI-MS strategy, at least 93 different components were detected in the *O. cayaporum* venom. Most of these components were also found by using the MALDI-TOF-MS strategy, which resulted in a total of 221 different molecular masses. In Table 1 are listed all molecular masses identified by mass spectrometry. They were grouped according to various time intervals corresponding to the retention time (RT) in the column of the

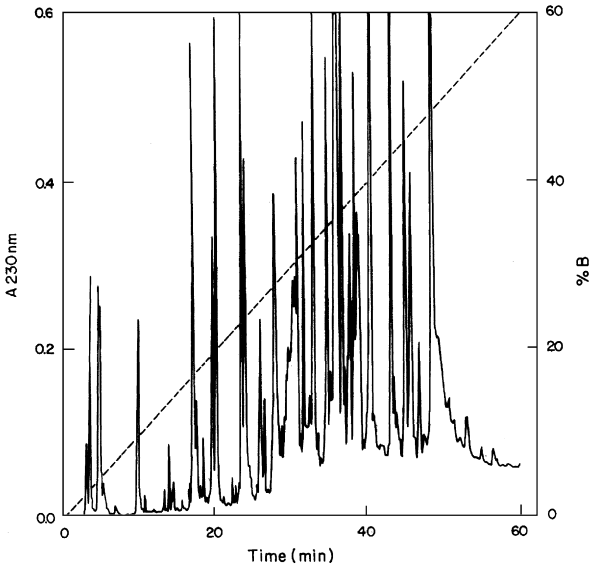

Fig. 1. RP-HPLC separation of *O. cayaporum* venom. Soluble venom (1 mg protein) from *O. cayaporum* scorpion was separated in a C18 reverse-phase column, eluted with a linear gradient from 0% solution A (0.12% TFA in water) to 60% solution B (0.10% TFA in acetonitrile) run at a flow rate of 1 ml/min, for 60 min duration.

HPLC system. The molecular masses vary from 229.2 (RT 14 min) atomic mass units (a.m.u.) up to 61,144.0 a.m.u. (RT 55.04 min). Analysis of the molecular weights obtained by mass spectrometry mapping of *O. cayaporum* venom reveals the presence of at least three groups of molecular mass components (Fig. 2), with 42% of the components possessing 229.2–2985.3 Da and 37% within the range of 3045.0–7258.6 Da. A third minor group distributes from 7458.4 to 9429.0 Da, with about 12%. This result also shows that the majority of components in *O. cayaporum* are short peptides containing approximately 40–45 amino acids residues, since about 60% of the components have molecular masses no higher than 5 kDa. The most abundant fractions were further separated by HPLC and 17 different peptides were obtained in homogeneous form and were submitted to automatic Edman degradation for the determination of the N-terminal amino acid sequences. A segment of sequence having from 7 to 31 amino acid residues were unequivocally identified for these 17 peptides, as shown in Fig. 3.

Usually, HPLC separation of scorpion venoms in a C18 reverse-phase column with a linear gradient form 0% to 60% acetonitrile run for 60 min shows that peptides eluting within the first 20 min correspond to low molecular weight components. Most of these compounds are thought to correspond to either short peptides or degradation products of others venom components (Pimenta et al., 2001; Batista et al., 2007). Components eluting from 20 to 30 min correspond to peptides of about 2500–3800 Da. In this range of molecular mass are included some of the α-KTxs, scorpion toxins specific for K^+ channels. From the *O. cayaporum* venom it was possible to isolate seven peptides within these molecular masses range. Their N-terminal sequences were determined. An important sequence similarity was found among several of these peptides, such as component eluting at RT 19.98 (17.84), 21.22 (18.81), 21.22 (24.34) and 21.73 (19.92). These components were separated twice. The value within parentheses indicates the time of elution from a second run on the HPLC starting with the material eluted in the first one. The peptide at RT 21.73 (19.92), for which the longer sequence was obtained, shows 67% identity to the α-KTx 6.10 toxin (OcKTx5) whose precursor was described in the scorpion *Opistophthalmus carinatus* belonging to the family of Scorpionidae (Zhu et al., 2004b). The venom of this scorpion (*O. carinatus*) has five different predicted sequences (called OcKTx1-5) which share a 47–97% identity with each other. In the venom of *O. cayaporum* the peptide eluting at RT 25.93 (30.06), whose molecular mass obtained was 3135.0 a.m.u., presents 61% identity to the Om-toxins from *O. madagascariensis* (Chagot et al., 2005). These toxins possess a structural motif characterized by two α-helical structures stabilized by two disulfide bridges, a fold only described previously for k-hefutoxin1 from the scorpion *Heterometrus fulvipes* (Srinivasan et al., 2002) and for BgK and ShK toxins from sea anemone (PDB codes 1BGK and 1ROO, respectively). Om-toxins and k-hefutoxin1 are blockers of the voltage-gated K^+ channels, Kv1.2 and Kv1.3. The segment of sequence corresponding to positions 6–13 (CSGSKRQK) of the peptide eluting at RT 18.34 (20.89) presents 87% identity to the 6–13 region (CSGSCRQK) of the OsK2. This was called α-KTx 13.2, and was shown to be an inhibitor of Kv1.2 channels identified in the venom of the scorpion *Orthochirus scrobiculosus* (Dudina et al., 2001).

3.2. Physiological assays, antimicrobial and enzymatic activities of several components

Most scorpion components eluting at RT interval from 32 to 45 min are toxins specific for Na^+ channels, which are the most abundant components of the Buthidae scorpion venom, being responsible for the lethality of these venoms. In *O. cayaporum* venom at least five peptides found by ESI-MS are thought to be Na^+-channels modulators: 6188.0, 6525.0, 7833.0, 7927.0 and 7928.0. Many others were observed by MALDI-TOF-MS. The RT 33.43 (29.03) (6525.0 a.m.u.) presents 50% identity to the probable neurotoxin precursor pcD-1008 from *Androctonus australis* (Q9BLM2).

The soluble venom of *O. cayaporum* was able to reduce the APs of cockroach nerve chord at low concentrations (0.22 mg/ml), and in a dose-dependent manner (Fig. 4). The ED_{50} obtained on the insect nerve assay was 1.1 mg/ml. At this same concentration, no effect was observed when the venom was applied to the rat nerve preparation (data not shown). Only a small reduction in the AP was induced when assayed at 3.11 mg/ml. This result is consistent with the observation that human accidents caused by Ischnuridae scorpions are not clinically important (Lourenço, 1981). From these results we conclude that the peptides specific for Na^+ channel present in *O. cayaporum* venom are responsible for the neurotoxic effect observed on cockroach nerves, but they might be

Table 1
Mass fingerprint of the *O. cayaporum* soluble venom

RT (min)	MM [M+H]$^+$	RT (min)	MM [M+H]$^+$	RT (min)	MM [M+H]$^+$	RT (min)	MM [M+H]$^+$	RT (min)	MM [M+H]$^+$	RT (min)	MM [M+H]$^+$
10–16	229.2	21–23 (cont.)	3750.3	29–33 (cont.)	**5050.4**	37–42	1002.4	37–42 (cont.)	6581.1	43–48 (cont.)	4656.6
	243.3		3790.7		5115.9		1198.6		6730.5		4735.3
	264.2		**3800.0**		5424.6		1308.7		6957.0		4848.4
	306.2		**3807.0**		5553.2		1334.9		7088.0		4928.6
	314.3		**3874.0**		5951.9		1351.6		7258.6		5063.3
	331.4		**3875.0**		6033.6		1357.0		7458.4		5300.4
	365.1		3986.0		**6188.0**		1386.8		7553.6		5357.7
	468.1		465.4		6225.8		1503.2		7726.1		5362.2
	630.5		**755.8**		6351.2		1526.6		7846.1		5379.9
	691.3	24–28	790.2		6467.2		1833.0		8004.6		5471.2
	714.6		**817.5**		**6525.0**		1858.0		8150.5		5497.8
	727.3		860.7		6560.7		1928.4		8267.1		5793.2
	737.3		903.6		6763.0		2030.3		**8315.0**		8298.9
	749.3		945.6		7833.0		2287.5		**8332.0**		8473.4
	917.6		1047.5		**7927.0**		2418.7		8614.8		8596.3
	995.7		**1098.6**		8221.5		2521.1		8701.8		8764.6
17–20	306.2		1196.5		8752.9		2589.3		10447.3		8804.3
	402.3		1217.7		**8772.0**		**3045.0**		10978.2		8872.7
	487.3		1254.5		**9136.0**		**3119.0**		**11370.0**		**8929.0**
	743.5		1472.8		**9282.0**		**3241.5**		12022.8		8990.1
	810.4		2266.2		9404.0		3721.8		12539.4		9294.6
	1371.5		2985.3		**9429.0**		3746.0		14117.0		10025.0
	1433.8		**3116.8**	34–36	930.3		3985.0		**14237.0**		12133.7
	1441.1		**3135.0**		980.5		**4072.3**		**14518.0**		17226.0
	2189.0		**5093.0**		**1086.8**		**4272.6**		14863.7		31256.0
	2883.0		5137.6		**1173.7**		**4457.1**		15050.8		44726.0
	3479.5		**5652**.0*		1210.8		**4668.0**		15204.2	49–60	1664.1
	3773.0		5818.9		1248.6		4765.8		16627.6		**4679.0**
21–23	766.9		999.7		**1285.6**		4881.0				4701.6
	818.5	29–33	1098.7		1408.9		4943.3	43–48	1158.5		4838.9
	980.5		1238.5		1451.8		5007.9		1268.2		4879.7
	1026.7		1390.7		1655.0		5226.8		1287.8		4900.5
	1074.4		1432.8		1735.7		5548.0		1545.8		5273.9
	1182.7		1565.0		2083.3		5620.2		**1561.9**		5330.7
	1227.9		1588.9		2300.9		6051.7		**1615.7**		5401.8
	1341.3		1801.0		4642.2		6179.0		1868.1		5817.0
	1467.9		1875.2		4794.1		6224.5		2054.3		6110.7
	1496.6		1963.0		4908.0		6302.6		2182.3		8934.3
	1517.9		2137.0		5258.7		6360.2		2320.3		17853.9
	1537.7		**2248.2**		5434.4		6466.2		**2535.4**		28037.0
	1633.0		**2545.2**		**5698.4**				3226.7		42479.0
	1903.3		**2702.0**		5856.4				3358.1		53226.0
	3571.0		4297.2		6121.9				3837.7		61144.0
	3732.0		4364.4		6611.2						
	3738.2		4974.6		6692.8						
					8558.0						
					8973.3						

This table shows the intervals of elution time of the various components as obtained in Fig. 1 and the corresponding molecular masses as determined by both ESI-MS and MALDI-TOF-MS, indicated by [M+H]$^+$. Molecular Masses (MM) in bold correspond to components observed by both ESI-MS and MALDI-TOF-MS. Underlined MM were found only by ESI-MS, but could not be confirmed by MALDI-TOF-MS analysis using the same chromatographic fraction.

specific for insects, having none or a negligible action on mammals.

Others components eluting around 40–45 min are the scorpine-like peptides (Conde et al., 2000; Diego-García et al., 2008). In *O. cayaporum* venom, the peptide eluting at RT 39.87 (31.86) presents 100% identity in the first 14 amino acids to *Pandinus imperator* and *Heterometrus laoticus* scorpines. The RT 39.87 (31.86), named here Ocy39.87, inhibited in 78 ± 2% the growth of *S. aureus* at 1.8 μM.

The chromatographic fractions RT 37.75 and RT 39.04 contain 14 kDa proteins, typical molecular mass of phospholipases. For these reasons they were assayed for possible phospholipasic activity, using the egg yolk-agarose assay. Both fractions, in fact, showed to have phospholipase activity (data not shown). The peptide Ocy37.75 was obtained in homogeneous form and had its N-terminal sequence determined. When submitted for blastp search against nr database, no homologous protein was detected considering the cut-off *e*-value of $<10^{-5}$. The fraction RT 39.10 was further separated by RP-HPLC resulting in two main fractions (data not shown), but both were positive for phospholipase activity. One of them was submitted to N-terminal sequencing and the resulting

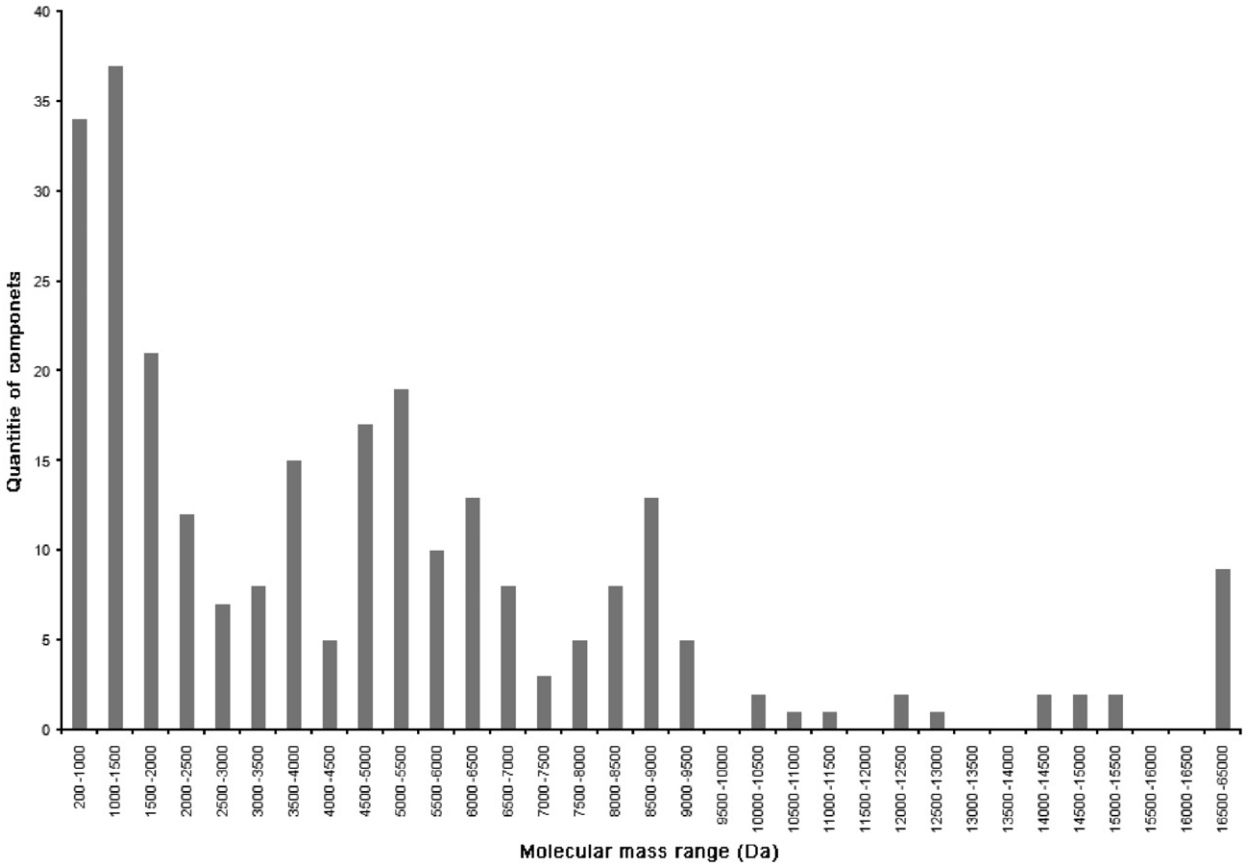

Fig. 2. Peptide mass distribution of the *O. cayaporum* chromatographic fractions obtained by ESI-MS and MALDI-TOF-MS. Histograms represent the frequency of MW distribution in 500 Da classes for 263 measured peptide masses, showing a trimodal molecular weight distribution, with 42% of the components possessing 229.2–2985.3 Da, 37% within the range of 3045.0–7258.6 Da and 12% within the range of 7458.4–9429.0 Da.

RT (min)	MW (Da)	Sequence
18.34 (20.89)	2188.0	SCKRECSGSKRQK...
19.98 (17.84)	3773.0	IRCTGSKECYSPSY...
21.22 (18.81)	3807.0	IRCQGSNQCYGHCREKTGCPNGKCID...
21.22 (24.34)	3800.0; 3571.0	IRCTGSKECYSPCYKATGCPNAKC...
21.73 (19.92)	3875	IRCQGSNQCYGHCREKTGCMNGKCINRVCKC...
21.73 (19.25)	3874	XNPELRCGLKD...
21.84(26.85)		DNLKKRRRCADDD...
25.50 (18.95)	5093.0	NRTFKTNTKCHVKNQCNFLCQ...
25.93 (30.06)	3135.0	YDACVNACLENHPNVRECEEAC...
27.64	1217.7	LGKSVTN...
35.82 (33.22)		GWGRTIRTHXSIDLNXGE...
33.43 (29.03)	6525.0	FENEDEGYFQDPEDCS...
37.75 (39.04)	14237.0	DFTGVKFDNTIGCGKG...
Oc2 32.49	14117.0; 14518.0	FMKVIDPGTKWCGPGNKAADDTDNGKN...
39.87 (31.86)	8315.0	GWINEEKIQKKIDEP...
40.78 (22.97)		AKNSAEANENSXDANE...
48.89	8929.0	GSLGEKYAQKAAEVLTSIIP...

Fig. 3. N-terminal amino acid sequences of peptides purified from *O. cayaporum* venom. The N-terminal amino acid sequences of 17 peptides/proteins from *O. cayaporum* are listed, according to their elution times from the HPLC column indicated RT (in between parenthesis second chromatography when it is the case) and their corresponding molecular masses (second column).

sequence was searched against database. This N-terminal sequence posses the conserved domain cd04704, characteristic of the sub-family of phospholipase A2, found in bee venom, and presented sequence similarities to other arthropod phospholipases, including the one isolated from *Heterometrus fulvipes* scorpion (AAZ78243).

Components eluting after the RT 45 min are more hydrophobic and usually correspond to high molecular

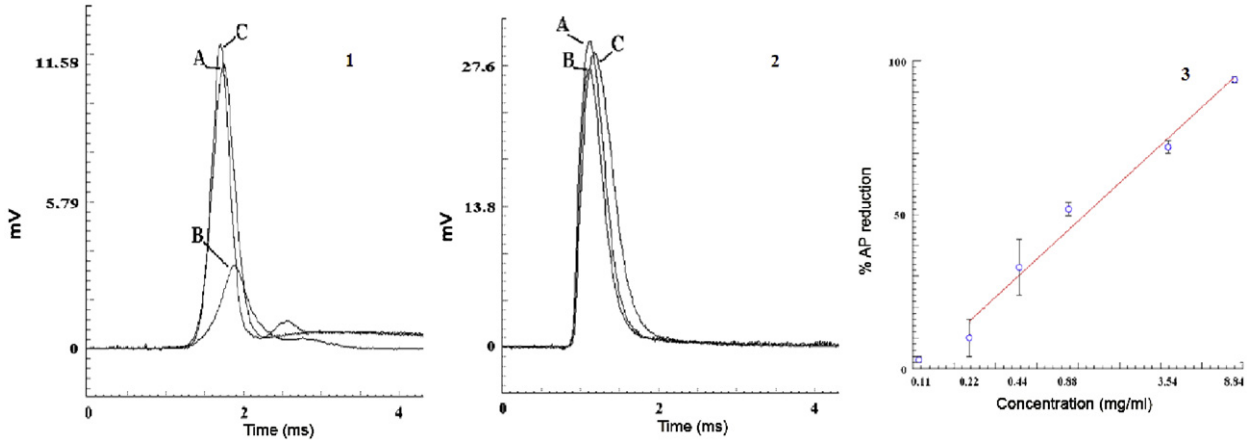

Fig. 4. Single sucrose-gap using cockroach's isolated ventral nerve chord (1) and rat sciatic nerve (2). After establishing the gap, the proximal end of the nerves received supramaximal stimulation (6–7 V) using a S8 stimulator (Grass Instruments. (1) 3.54 mg/ml of the crude venom from *O. cayaporum*; (2) 3.11 mg/ml of the crude venom. (A) Control; (B) 25 min after adding venom; (C) reversion of the blockage effect after 30 min washing with saline solution. (3) Dose–response curve of *O. cayaporum* crude venom obtained on cockroach's isolated ventral nerve chord. The values are presented as average ± mean error. $n = 3$ in (mg/ml) 0.11; 0.22; 0.44 and 0.88; and $n = 2$ in (mg/ml) 3.54 and 8.84. The curve was linearized using a monolog function (the R correlation obtained was 0.9892). ED50 = 1.1 mg/ml.

weight proteins (Batista et al., 2007). In the venom of *O. madagascariensis* a peptide named IsCT was isolated (Dai et al., 2002) around RT 45 min. It was demonstrated to be an antimicrobial peptide with 1502 Da which shows an amphipathic α-helical structure. Table 1 shows the presence of peptides with similar molecular masses (1545.8, 1561.9 and 1615.7 a.m.u.) in the venom of *O. cayaporum*. They also elute around RT 45 min and they are surmised to be hydrophobic antimicrobial peptides.

Two proteins with molecular weights of about 42–44 kDa elute at the RT of 47–49 min, as observed in other scorpion venoms, and characterized as hyaluronidases (Batista et al., 2007).

3.3. Gender variation of venom components

The peptide IsTX isolated from *O. madagascariensis* is a potassium channel blocker peptide described as a sex-specific peptide by Yamaji et al. (2004). In order to verify the existence of sex-specific molecular mass difference in *O. cayaporum* venom, male and female venoms were separated, pooled and submitted to HPLC fractionation. The chromatographic profiles are very similar as shown in Fig. 5. Although using exactly the same amount of each venom, some differences in absorbance amplitude of six fractions were observed. The whole crude venoms of male and females were also analyzed by MALDI-TOF mass spectrometry. Since venoms are complex mixtures whose individual compounds may have different ionization efficiency and also because of possible suppression effects, each venom sample was analyzed in two concentrations (1 and 10 μg) and each one of them in triplicate. The acquisition data ranges from 600 to 40,000 Da. The results obtained under these experimental conditions showed that each MALDI-TOF MS experiment was highly reproducible. The female and male spectra were qualitatively very similar. The most significant differences were the variation of intensity of some peaks. Fig. 6 shows the acquisition data from 1000 to 10,000 Da range, and illustrates these quantitative differences. About 56 peptides were found in both female and male spectra, in both concentrations. All these components had already been identified by mass spectrometry analysis of HPLC fractions as described before. Fewer compounds were found in the intervals up and down the 1–10 kDa range.

The most evident quantitative differences were the 4909 and 7929 Da peaks, being more abundant in female venom, and 8315 and 8930 Da peaks, which were more abundant in male venom. A 4908 Da peptide was found in the fingerprinting analysis conducted with the chromatographic fractions on the MALDI-TOF-MS, but we could not further characterize it at this time. The 7929 Da peak could correspond to the RT 32.24 or 32.82 min peptide, and as discussed before, could be a Na^+-channel modulator. The 8315 Da peak corresponds to the scorpine-like peptide, as already mentioned. We assumed that the 8930 Da peak corresponds to the RT 48.89 (8929 Da), whose N-terminal sequence has sequence similarities to that of the β-KTx, peptides that were characterized recently (Diego-García et al., 2008).

3.4. Concluding remarks

This is the first complete report on the general characterization of the venom of the Ischnuridae scorpion *ls, but is active on insects. The venom is composed of more than 250 different components as determined by the mass spectrometry analysis. Among the components clearly identified are peptides similar to K^+-channel- and Na^+-channel-specific toxins reported to be present in other scorpions of the family Buthidae. At least two components with positive phospholipase activity and one with clear antibacterial activity were identified. Partial N-terminal amino acid sequence determination of 17 of these components showed*

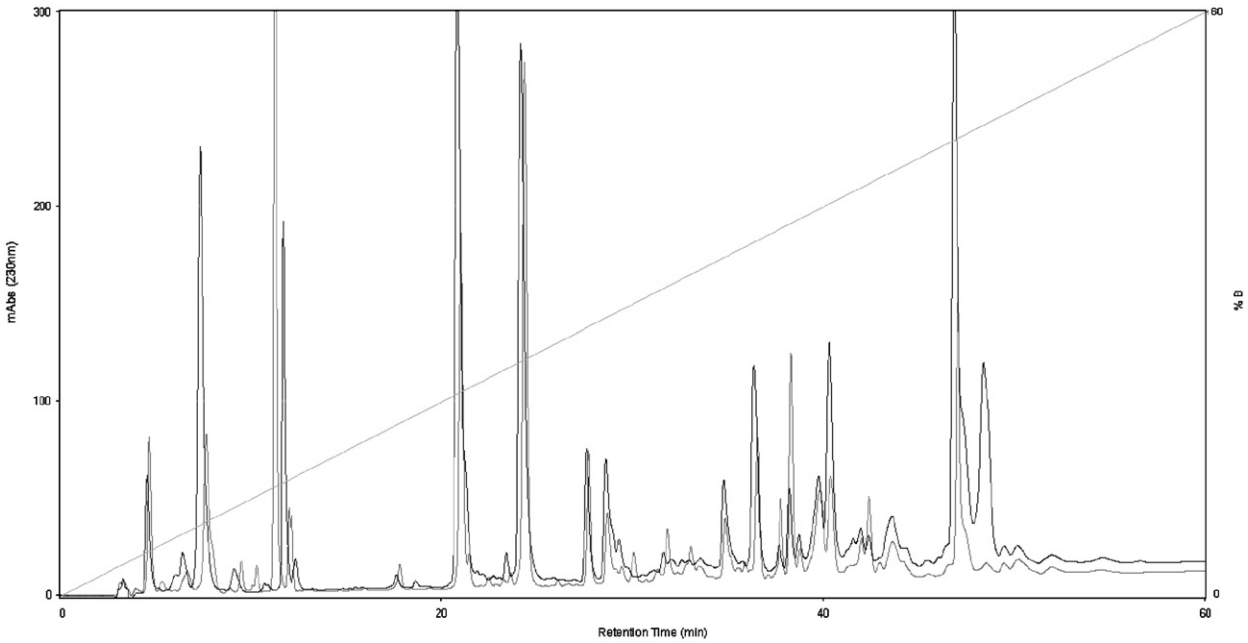

Fig. 5. RP-HPLC separation of *O. cayaporum* male (black) and female (gray) venom. Soluble venoms (1 mg protein) were separated in a C18 reverse-phase column, eluted with a linear gradient from 0% solution A (0.12% TFA in water) to 60% solution B (0.10% TFA in acetonitrile) run at a flow rate of 1 ml/min, for 60 min duration.

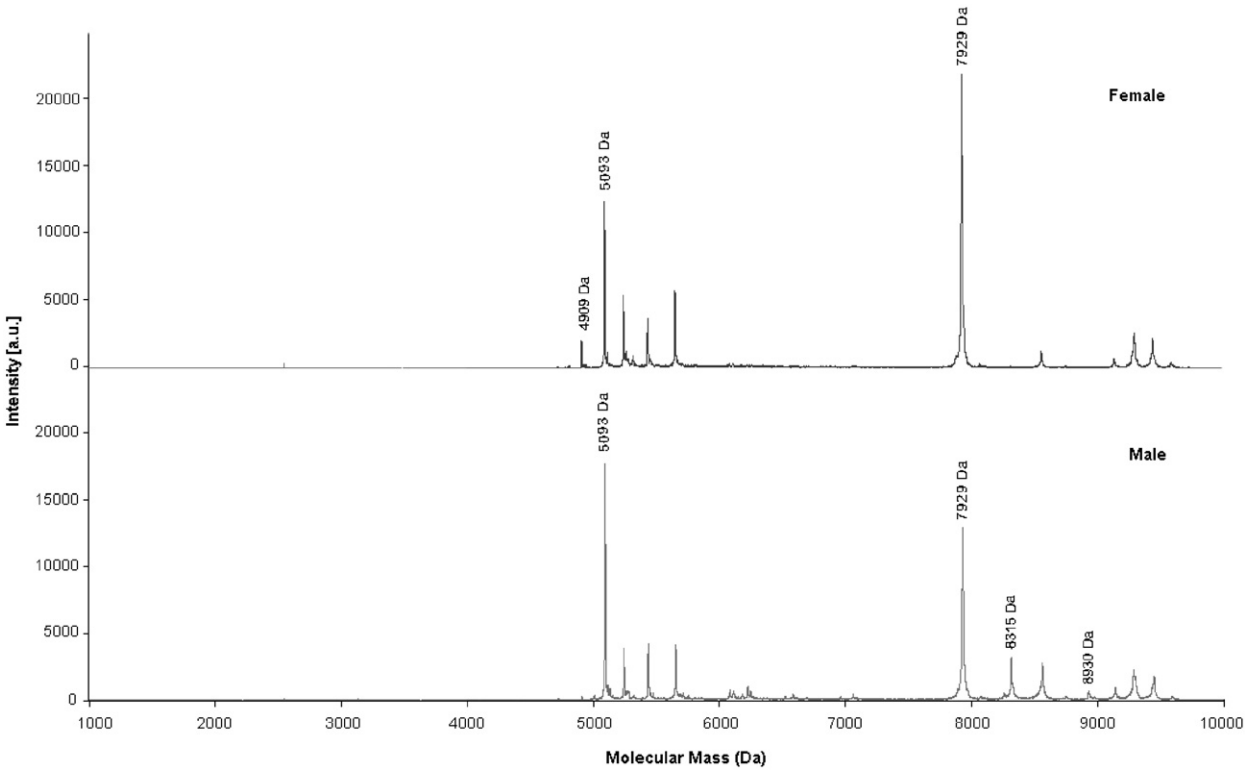

Fig. 6. MALDI-TOF mass spectra of *O. cayaporum* venom (acquisition data from 1000 to 10,000 Da range). The figure shows the results obtained with 1 μg sample, as a function of the scorpion gender: (upper trace) female and (bottom trace) male scorpions. Only quantitative differences were observed. Components with masses 8315 and 8930 Da showed a higher concentration in male venom, and the peptides with masses 4909 and 7929 Da occurred in a higher concentration in female. Using this approach no qualitative molecular mass differences were observed between both samples.

some sequence similarities with scorpions of other related species. It is not clear whether there is significant gender differences on animals of this species. These results open the field for additional research, especially for the unidentified components of the venom, as shown here.

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