

High-resolution proteomic profiling of spider venom: expanding the toxin diversity of *Phoneutria nigriventer* venom

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Abstract Here we present a proteomic characterization of *Phoneutria nigriventer* venom. A shotgun proteomic approach allowed the identification, for the first time, of *O*-glycosyl hydrolases (chitinases) in *P. nigriventer* venom. The electrophoretic profiles under nonreducing and reducing conditions, and protein identification by mass spectrometry, indicated the presence of oligomeric toxin structures in the venom. Complementary proteomic approaches allowed for a qualitative and semi-quantitative profiling of *P. nigriventer* venom complexity, expanding its known venom proteome diversity.

Keywords *Phoneutria nigriventer* · Proteome · Spider venom · Chitinase

Introduction

Spider venoms are rich sources of bioactive compounds whose chemical scaffolds have the potential to serve as basis for the development of pharmacologically active molecules (Escoubas et al. 2000; Escoubas 2006; Bosmans et al. 2008; Nunes et al. 2013; Baron et al. 2013; Pineda et al. 2014). In addition to the remarkable potential for drug development, spiders are also of great medical importance as one of the main group responsible for human accidents worldwide (Diaz 2004). In this context, the Brazilian “armed” spider, *Phoneutria nigriventer* (Ctenidae) is a widely distributed species, ranging from Central through South and Southeastern states of Brazil (Lucas 1988). Human accidents with spiders belonging to *Phoneutria* genus are complex, with local and systemic effects (Bucarety et al. 2000) and, according to recent reports, they account for almost one-third of the total notification cases in Brazil (SINAN/SVS, Brazilian Ministry of Health 2015). One of the main biological effects of *P. nigriventer* venom is the neurotoxic activity, which is strongly related to toxins that target voltage-gated ionic channels (Gomez et al. 2002; Troncone et al. 2003; Gomes et al. 2011; Kalia et al. 2015). The venom proteome of *P. nigriventer* was previously assessed by two-dimensional (2DE) gel electrophoresis and mass spectrometric approaches and showed a number of post-translationally modified peptides as well as cationic low-molecular weight compounds, including some structurally and biologically related to the venom of two congeneric species, *P. reidyi* and *P. keyserlingi* (Richardson et al. 2006; Pimenta et al. 2005).

As of its last release, UniProt database comprises 85 protein entries related to *Phoneutria* genus, including 58 of *P. nigriventer*, 12 of *P. keyserlingi*, 11 of *P. reidyi*, 3 of *P. fera* and 1 of *P. pertyi* (release 2015_10; www.uniprot.org). However, as previously reported, the actual diversity of toxins

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derived from *Phoneutria* spiders might vary tremendously, mainly due to post-translational proteolytic processing/modifications (Richardson et al. 2006; Pimenta et al. 2005; Penaforte et al. 2000; Kalapothakis et al. 1998; Diniz et al. 1993). Therefore, in order to explore *P. nigriventer* toxin diversity, we employed two complementary proteomic approaches based on (1) *in solution* trypsin digestion of crude *P. nigriventer* venom followed by high-resolution LC–MS/MS analysis and (2) SDS–polyacrylamide gel electrophoresis under reducing and non-reducing conditions, followed by *in-gel* trypsin digestion and LC–MS/MS analysis.

Materials and methods

Phoneutria nigriventer venom

Phoneutria nigriventer venom (manually extracted) was provided by Arthropod Laboratory from Instituto Butantan, São Paulo, Brazil. Protein concentration was determined using the Bradford reagent (Sigma) and bovine serum albumin (Sigma) as a standard.

Shotgun proteomic analysis of *P. nigriventer* venom

One-hundred micrograms of *P. nigriventer* venom proteins was submitted to *in-solution* trypsin digestion, according to the protocol described by Kleifeld et al. (2011). An aliquot (10 µg) of the resulting peptide mixture was analyzed by high-resolution mass spectrometry using an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, USA). The mass spectrometer was operated in data-dependent mode, in which one full MS scan was acquired in the m/z range of 300–1650 followed by MS/MS acquisition using collisional-induced dissociation (CID) of the ten most intense ions from the MS scan. MS spectra were acquired in the Orbitrap analyzer at 30,000 resolution (at 400 m/z). Dynamic exclusion was defined by a list size of 500 features and exclusion duration of 90 s. Peak lists obtained from MS/MS spectra were identified using X!Tandem (v. 2013.09.01.1) (Craig and Beavis 2004), MS-GF+ (v10282) (Kim and Pevzner 2014) and Comet (v.2015.02 rev. 1) (Eng et al. 2013). The search was conducted using SearchGUI (2.1.4) (Vaudel et al. 2011) with a false discovery rate $\leq 1\%$ at both peptide and protein levels. A semi-quantitative analysis was carried out using spectral counting of all spectra associated with the identified amino acid sequences.

In-gel trypsin digestion and mass spectrometric analysis of *P. nigriventer* venom

For protein identification, protein bands were submitted to *in-gel* trypsin digestion (Hanna et al. 2000) and the resulting

Fig. 1 Qualitative and semi-quantitative diversity of *P. nigriventer* venom proteome. **a** Semi-quantitative profile of *P. nigriventer* venom proteome (after excluding tryptic peptides derived from hemocyanin) obtained by spectral counting associated with the identified amino acid sequences corresponding to known toxins and other spider proteins. **b** SDS-PAGE (8–18 % SDS–polyacrylamide gel) profile of *P. nigriventer* venom (50 µg). Mobility of molecular mass markers is indicated on the left. The arrows indicate bands of proteins identified by mass spectrometry. Proteins were stained with Coomassie blue. **c**, **d** MS/MS spectra derived from tryptic peptides from the identified chitinases (UniProt #Q8ISH5 and A0A087SXZ2, respectively); precursor ions of m/z of 723.88²⁺ (**c**) and m/z of 791.33²⁺ (**d**). Spectra obtained by PeptideShaker software (Vaudel et al. 2015) (for details on protein identification, see Supplementary Tables 1–3)

peptide mixture (4.5 µL) was analyzed by LC–MS/MS using a nanoUPLC (nanoAcquity, Waters, Milford, MA) coupled to a Quadrupole-Time of Flight mass spectrometer (Q-TOF Ultima, Waters, Milford, USA) as described elsewhere (Zelanis et al. 2010). For both shotgun and *in gel* trypsin digestion approaches, database searching was carried out using the last release of the non-redundant UniProt database concerning the taxonomical class Araneae (downloaded on 6 November 2015, 48,986 entries) using the open source software X!Tandem (Craig and Beavis 2004).

Results and discussion

After excluding tryptic peptides derived from the hemolymph contaminant hemocyanin, shotgun proteomic analysis allowed for the identification/quantification of previously reported protein toxins in *P. nigriventer* as evidenced by U21-ctenitoxin-Pn1a, Techylectin-like protein, U10-ctenitoxin-Pk1, U2-ctenitoxin-Pn1a, U21-ctenitoxin-Pn1a, Delta-ctenitoxin-Pn2c and U2-ctenitoxin-Pn1b, which accounted for more than 70 % of the identified proteins (Fig. 1a, Supplementary Tables 1 and 2). Among the remaining ~30 %, a number of proteins which may display a role in *P. nigriventer* venom biological activities were also identified, including two *O*-glycosyl hydrolases (chitinases), Mite allergen Lep d 7 and a putative apolipoprotein (cysteine-rich protein). As previously reported by Richardson et al. (2006), the number of protein/peptides in *P. nigriventer* venom might be higher than 150; therefore, it is reasonable to suggest that the toxins identified herein are among the most abundant toxins of *P. nigriventer* venom and their tryptic peptides have suppressed the signal of the low abundant tryptic peptides (derived from toxins whose abundance were lower). The majority of venom tryptic peptides eluted within the first 40 min of LC–MS/MS run, at a concentration of ~30 % of acetonitrile, and displayed a net charge of +2; however, approximately 33 % of the analyzed isotopic clusters displayed charge states of +3 and +4 (Supplementary Fig. 1).

Table 1 Mass differences (predicted vs observed) among the identified protein bands indicated in Fig. 1B by LC–MS/MS analysis

Band #	Identified protein(s)	MM predicted (kDa) ^a	MM observed (kDa)	Mass difference (kDa)
3	Techylectin-like protein	25.7	55	29.3
4	U10-ctenitoxin-Pk1a	16.5	48	31.5
6	U21-ctenitoxin-Pn1a	26.7	31	4.3
7	U10-ctenitoxin-Pk1a	16.5	50	33.5
11	Techylectin-like protein	25.7	32	6.3
	U21-ctenitoxin-Pn1a	26.7	32	5.3
12	U21-ctenitoxin-Pn1a	26.7	30	3.3
13	U20-ctenitoxin-Pn1a	8.8	14	5.2
14	Delta-ctenitoxin-Pn2a	5.2	8	2.8
	Omega-ctenitoxin-Pn3a	8.7	8	−0.7
15	Kappa-ctenitoxin-Pn1a	4.5	6	1.5

^a Mature form (according to UniProt: www.uniprot.org)

Although our shotgun approach has not allowed for the profiling of low molecular weight compounds (i.e. natural peptides), here we report, for the first time, on the presence of chitinases in the venom of *P. nigriventer* (Fig. 1c, d). Such enzymes might display an important role in *P. nigriventer* feeding habits, since chitin is the main component of arthropod exoskeletons. The presence of this protein class seems to be a recurrent feature in spider venoms, as recently reported by Haney et al. (2014) in a comprehensive study on *Latrodectus hesperus* venom.

In order to obtain a complementary view of *P. nigriventer* venom complexity, we performed a decomplexation method prior to proteomic analysis, through SDS–polyacrylamide gel electrophoresis under reducing and non-reducing conditions. The electrophoretic profile of *P. nigriventer* venom was rather distinct under reducing and non-reducing conditions, as observed in Fig. 1b, indicating the presence of proteins containing interchain disulfide bonds. Interestingly, under reducing conditions the venom showed major protein bands of 6–14 kDa which were also visible as major spots on a 2DE profile previously reported (Richardson et al. 2006). The major protein band whose profile did not change in both conditions was a hemolymph contaminant, identified as hemocyanin, at ~60 kDa. Hemocyanin peptides were also identified in almost all of the sampled protein bands and by shotgun proteomic analysis indicating that this contaminant protein, which is present in our venom sample due to the process of manual extraction, and was also reported in other studies on arthropod venoms (Oukkache et al. 2013), may have been partially degraded by venom proteolytic enzymes (Supplementary Table 3). A total of 15 protein bands were subjected to *in-gel* trypsin digestion and LC–MS/MS analysis. Of interesting note was the fact that a number of identified proteins differed from their predicted molecular masses, as illustrated in Table 1. The striking differences were found for techylectin-like protein (UniProt ac.# P85031) and

U10-ctenitoxin-Pk1a (UniProt ac.# P85860), whose molecular masses differed in (+)29 kDa and (+)31 kDa, respectively. Since these proteins were identified in gel bands of venom electrophoresed under non-reducing conditions, it is reasonable to suggest that in their native form they might be covalent oligomers, linked through disulfide bonds. On the other hand, in the UniProt database both proteins are stated as ‘fragments’ of the actual toxins. Therefore, their ‘full’ versions might exist as higher molecular mass protein species, either linked to another peptide(s) chain(s) through disulfide bond(s) or with a yet unknown peptide extension. In fact, in both toxins the primary structure available at UniProt comprises an odd number of cysteine residues, which implies in a free sulfhydryl group (–SH) that could potentially bridge to another polypeptide chain via disulfide bond with a free –SH group. In the case of techylectin-like protein, only two disulfide bonds are annotated at UniProt (Cys41–Cys60 and Cys172–Cys185, entry # P85031). Moreover, mass differences were also found among the low molecular mass bands (#13–15) containing ctenitoxins (Fig. 1b). In this respect, *O*-glycosylation sites were predicted using NetOGlyc server (<http://www.cbs.dtu.dk/services/NetOGlyc/>; version. 4.0) for U-20 ctenitoxin-Pn1a, at Thr65, Ser66 and Thr72 and at Thr36 for Delta-ctenitoxin-Pn2a. Therefore, glycan chains might account for the observed mass differences.

The two approaches used in this work allowed for a complementary view of the proteome diversity of *P. nigriventer* venom. In fact, comprehensive databases related to spider venom toxins are anything but ideal in terms of the number of protein/peptide entries, and this feature has a main impact on the search of data generated in high-throughput mass spectrometric analysis. In addition, a number of protein entries derived from spider venom/venom gland are actually ‘fragments’ or yet uncharacterized/hypothetical proteins. De novo analysis of MS/MS spectra and homology searches are valuable tools for the study of venom

toxins (Tashima et al. 2012; Pimenta et al. 2005; Zelanis and Tashima 2014). However, given the number of MS/MS spectra that can be generated even in a short LC–MS/MS run of venom tryptic peptides, large scale manual De novo sequencing becomes a less feasible task. As the number of protein entries in the main proteomic database grows—a direct result of high-throughput ‘omics’ approaches such as genomics and transcriptomics—also increases the likelihood of profiling proteomes of interest after database searching of mass spectrometry data, either by direct identification or through the identification homologous counterparts. Nevertheless, the use of high-resolution instruments is also of paramount importance, as it also increases the confidence in peptide/protein identification. Therefore, complementary proteomic approaches such as the ones we used in this study (i.e. *in gel*- and *in solution* trypsin digestion) allowed for a qualitative and semi-quantitative profiling of *P. nigriventer* venom, expanding its known venom proteome structural diversity.

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Compliance with ethical standards

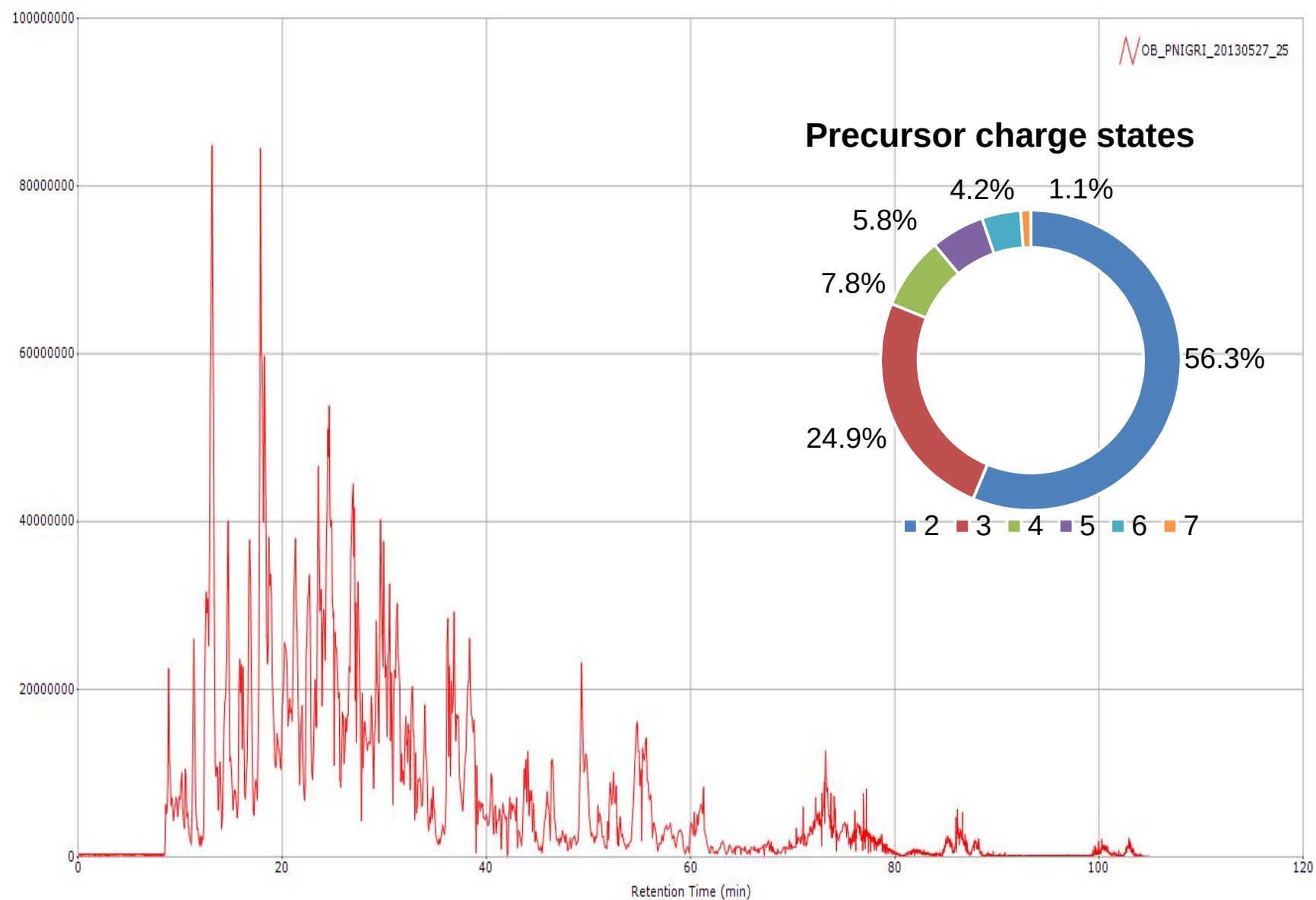
Conflict of interest The authors declare that they have no conflict of interest.

Human rights and animal statement This article does not contain any studies with human participants or animals performed by any of the authors.

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Supplementary Figure 1: Base peak intensity chromatogram derived from the LC-MS/MS run of 10 μ g of *P. nigriventer* venom tryptic peptides. Orbitrap Velos raw file was analyzed in RawMeat software (<http://www.vastscientific.com/rawmeat>). Inset: precursor charge states proportion among all submitted MS/MS.