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Combined peptidomic and proteomic analysis of electrically stimulated and manually dissected venom from the South American bullet ant *Paraponera clavata*

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Abstract

Ants have evolved venoms rich in peptides and proteins used for predation, defence and communication. However, they remain extremely understudied due to the minimal amount of venom secreted by each ant. The present study investigated the differences in the proteome and peptidome of the venom from the bullet ant, *Paraponera clavata*. Venom samples were collected from a single colony either by manual venom gland dissection or by electrical stimulation and were compared using proteomic methods. Venom proteins were separated by 2D-PAGE and identified by nanoLC-ESI-QTOF MS/MS. Venom peptides were initially separated using C18 reversed-phase high performance liquid chromatography then analysed by MALDI-TOF MS. The proteomic analysis revealed numerous proteins that could be assigned a biological function (total 94) mainly as toxins, or roles in cell regulation and transport. This investigation found that ca. 73% of the proteins were common to venoms collected by the two methods. The peptidomic analysis revealed a large number of peptides (total 309) but with <20% shared by the two collection methods. There was also a marked difference between venoms obtained by venom gland dissection from different ant colonies. These findings demonstrate the rich composition and variability of *P. clavata* venom.

Introduction

Ants of the order Hymenoptera are a diverse group of insects, with ca. 16,000 extant species belonging to 3 clades and 16 subfamilies^{1, 2}. The number of species within each subfamily ranges from 1 to more than 6000². Of these subfamilies, 13 comprise ants that employ a stinger to inject a peptide-rich venom used for predation, defence and communication³⁻⁶. Although hundreds of peptides and proteins, in each venom, bear those functions, little work has been undertaken to characterise these components in detail (for recent reviews see^{2, 7}).

Venom proteins and peptides are now routinely being investigated as drug or biopesticide leads, particularly those from bees⁸, spiders^{9, 10} and cone snails¹¹⁻¹⁴ as many of these organisms are of medical importance, which suggests interesting pharmacological properties. *Paraponera clavata* (commonly known as the bullet ant or giant tropical ant) is a stinging ant that belongs to the subfamily Paraponerinae of the poneroid clade^{15, 16}. It is one of the few ants of medical importance due to its extremely painful sting¹⁶⁻¹⁸ that is used to deter predators and capture prey¹⁹. *P. clavata* venom is toxic to both vertebrates and invertebrates and is thought to elicit at least part of its action through the neurotoxic peptide poneratoxin²⁰⁻²³. This toxin induces slowly activating Nav1.7 mediated currents at low activation voltages and also sustained sodium currents as a result of a slowing of Nav channel inactivation¹⁶, similar to that described previously in rat and frog skeletal muscle²³. All studies performed on this ant thus far have focussed on this peptide, as it is seen as a potential peptide for commercialisation as an insecticide due to its high activity²⁰. An example of other ant-venom derived peptides that have been thoroughly investigated are poneritoxins from the ant *Neoponera goeldii* (Ponerinae; formerly *Pachycondyla goeldii*). These were found to have antimicrobial and insecticidal activities²⁴. These peptides confirm that the venom of ants can be potential source of bioactive peptide leads that can be developed into therapeutic or insecticidal drugs.

To date the major hurdle for studying ant venoms has been the limited amount of venom present in their venom glands, however, this restriction has been overcome with recent advances in mass spectrometry and DNA sequencing techniques²⁵. Ant venom is conventionally obtained by venom gland dissection, however it can also be collected by

electrical stimulation, which can be considered a more efficient method of collection since ants remain alive following venom collection. These two collection methods have been employed with spider²⁶, cone snail¹³, wasp²⁷, scorpion²⁸ and bee venoms³ and differences in the venom components have been seen between these two collection methods²⁹⁻³¹. The dissection technique has been used for many years for a variety of different arthropods after it was first introduced in 1961³². However, if isolation of only one certain venom component is desired, then electrical stimulation would be the better collection method, as it increases the likelihood of isolating the potent component due to the absence of contaminants such as structural proteins from the venom gland^{27, 33}. The other advantage of electrical stimulation, if performed en masse, is that it can give a higher yield of venom, is less time consuming and keeps the organism alive for subsequent venom extraction³⁴. If mass milking is not possible, as is the case with vespid venoms²⁷, then automated methods of milking individual venoms have also been proposed³⁵. Previous work suggests that dissected venom contains most of the proteins that are in electrically stimulated venom but also other proteins that are usually contaminants from the venom gland including cellular proteins used in metabolic machinery or toxin maturation and processing^{13, 27, 36, 37}. Electrically stimulated venom may therefore give a more genuine representation of the venom components. The present study aimed to compare the proteomic and peptidomic components of *P. clavata* obtained by electrical stimulation and manual dissection. While previous studies have looked at individual components of either the proteome or peptidome of ant venoms, this is the first in-depth study of both proteomic and peptidomic components. It also aimed to identify the presence of inter-colony variations to further confirm the diversity of this group of insects.

Experimental Section

Venom Collection

Venom samples from *P. clavata* were collected from two separate ant colonies at “la Montagne des Singes” near Kourou, French Guiana. To determine differences in venom profiles using the two collection procedures, ants from one colony (colony 1) were separated into two random groups and venom was extracted by either electrical stimulation or manual dissection of individual workers. To establish the extent of inter-colony variations

in *P. clavata* venom profiles, venom from another colony (colony 2) was also obtained by venom gland dissection only. The manually dissected venom gland samples were prepared by storing ants at $-20\text{ }^{\circ}\text{C}$ prior to the dissection of the venom glands. After dissection, the glands were pooled in 10% (v/v) acetonitrile (ACN)/water. Samples were then centrifuged for 5 min at 14,400 rpm (12,000 g_{av}), the supernatant was collected and lyophilized prior to storage at $-20\text{ }^{\circ}\text{C}$. Electrically stimulated venom was collected by placing individual *P. clavata* ants into a glass insert. Ants were then milked by placing a pair of tweezers attached to electrodes on their abdomen and a 12 V square wave pulse of 1 ms duration delivered at 100 Hz. The venom was collected into a glass tube insert and diluted with 100 μL of 10% ACN. The venom was then transferred to an Eppendorf tube prior to being freeze dried.

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

Lyophilized crude electrically stimulated or manually dissected venom was separated on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) using a Vydac analytical C18 RP-HPLC column (218TP54, 25 cm x 4.6 mm, 5 μm particle size; Grace, MD, USA). Fractionation of venom peptide components was achieved using a gradient of 0.085% (v/v) trifluoroacetic acid (TFA) in ACN (solvent B) in 0.1% (v/v) TFA in water (solvent A). Separation was achieved using a flow rate of 1 ml/min with the following gradient: 0–5 mins, 0% solvent B; 5–65 mins, 0–60% B; 65–75 mins, 60–90% B; 75–80 mins, 90% B; 80–85 mins, 90–0% B; and 85–90 mins, 0% B. Peaks were monitored at absorbances of 280 nm and 215 nm and manually collected, then fractions were lyophilised and stored at $-20\text{ }^{\circ}\text{C}$ for further analysis.

MALDI-TOF MS Analysis

MS analysis of each HPLC fraction was performed on an AB SCIEX TOF/TOF™ 5800 mass spectrometer (AB SCIEX, MA, USA). Each venom fraction was manually collected and freeze-dried, then reconstituted in 10 μL water. Subsequently 1 μL of each fraction was then spotted onto a MALDI plate and overlaid with 0.5–0.75 μL of matrix using the dried droplet method³⁸. Matrix consisted of 5 mg/ml of α -cyano-4-hydroxycinnamic acid (CHCA) dissolved in 1% (v/v) TFA / 100% (v/v) ACN / 0.1 M $\text{NH}_4\text{H}_2\text{PO}_4$ (45:45:10). Each spectrum was calibrated externally using a mixture of peptides with known molecular masses in the same m/z range (AB SCIEX). Mass spectra were acquired in positive linear mode over the range 1,000–10,000 m/z with 400 laser shots accumulated for each sample, based on the acceptance parameters

and adequate signal intensity. Signals below 1,000 m/z were not recorded as they are comprised of mostly matrix-related ion clusters³⁹. Data Explorer® v4.11 software (AB SCIEX) was used to analyse spectra from individual RP-HPLC fractions to characterise the number and masses of peptides per venom, as previously reported⁹. Briefly, spectra were subjected to baseline correction with a correlation factor of 0.7 and Gaussian smoothing to reduce noise with a 5-point filter width. All mass attributions were verified manually and a mass list created for each LC-MALDI-TOF MS run. Potential adducts from oxidation, hydration, sodium, and deamination were manually removed from all mass lists, as well as any potential dimers or doubly-charged species. Peptides with mass matches within ± 1.0 Da in adjoining HPLC fractions were considered identical and were removed from the data set as they likely reflect incomplete separation. For each venom, the peptides from each RP-HPLC fraction were consolidated into one mass list. Mass matching across data sets was performed in Microsoft Excel (Redmond, USA) to identify similar peptides across the different venoms. Two-dimensional scatter plots, termed "2D venom landscapes", were constructed using Prism v6 software (GraphPad, La Jolla, CA USA). All peptide masses detected in each HPLC fraction were plotted as a function of their m/z values (x -axis) and their HPLC retention time reflecting their hydrophobicity (y -axis). Area-proportional Euler plots depicting overlapping peptide masses or proteins from different venoms were constructed using eulerAPE software (www.eulerdiagrams.org/eulerAPE/).

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

300 μg samples of venom, collected by electrical stimulation or manual dissection, were re-suspended in 100 μl 7 M urea, 2 M thiourea, 1% (v/v) C7BzO, 50 mM Tris HCl pH 8.8 before reduction and alkylation of disulphide bonds with 5 mM tributylphosphine (TBP) and 20 mM acrylamide monomers for 90 mins. The sample was subjected to 2D-PAGE as previously described⁴⁰. Briefly, sample was fractionated by isoelectric focusing on an 11 cm pH 3–10 IPG strip (Bio-Rad, Hercules CA) for 100 kVh and then further separated by molecular size by SDS-PAGE on a 4–20% Tris-glycine gel (Criterion TGX, Bio-Rad, NSW, Australia). The gel was then placed in a fixing solution containing 40% (v/v) methanol and 10% (v/v) acetic acid for 30 mins at room temperature before staining with Flamingo fluorescent stain (Bio-Rad) for 1 h. After staining, the gel was scanned using a Molecular Imager PharosFX Plus system (Bio-Rad) with QuantityOne® software. In order to perform nanoLC-ESI-QTOF MS/MS, gels were

additionally stained with Coomassie Blue G250 overnight then destained with 1% (v/v) acetic acid for 2 h.

NanoLC-ESI-QTOF MS/MS Analysis

Gel spots or bands were excised and digested as previously described⁴¹. Peptide samples were then fractionated using a Tempo nano LC system (Eksigent, California, USA). Samples were first loaded onto a Michrom reversed-phase trapping cartridge at a rate of 20 μ l/min, then eluted onto a 75 μ m x 150 mm PicoFrit column (New Objective, MA, USA) packed with Magic C18AQ chromatography resin (Bruker-Michrom Biosciences, CA, USA). An increasing gradient of ACN at 300 nl/min eluted the peptides which were ionised at 2300 V by the MicroLonspray II head holding the PicoFrit column with integrated emitter into the source of a QSTAR Elite™ Quadrupole TOF mass spectrometer (AB SCIEX, MA, USA). An Intelligent Data Acquisition (IDA) experiment was performed, with a mass range of 350–1500 Da scanned for peptides of charge state 2⁺ to 5⁺ with an intensity of more than 30 counts/scan. The selected peptides were then fragmented, and the ion fragment masses were measured over a mass range of 50–1,500 Da. The mass of the precursor peptide was then excluded for 15 s.

Protein Identification

The MS/MS data files were searched using Mascot (v2.4.0) against the LudwigNR database. This database comprised of the UniProt, plasmDB and Ensembl databases (vQ114), provided by the Australian Proteomics Analysis Facility (APAF Ltd), hosted by the Walter and Eliza Hall Institute for Medical Research Systems Biology Mascot Server. The database was searched with the following parameter settings: fixed modifications, none; variable modifications, propionamide, oxidised methionine, deamidated asparagine and glutamine; enzyme, semi-trypsin; number of allowed missed cleavages, 3; peptide mass tolerance, 100 ppm; MS/MS mass tolerance, 0.2 Da; charge state, 2⁺, 3⁺ and 4⁺. PEAKS 7 software (Bioinformatics Solutions Inc., ON, Canada) was used to generate de novo sequences. A database comprising ant protein sequences available on the NCBI database and known contaminant proteins was compiled and uploaded onto PEAKS. De novo sequences were then also searched against this database. Remaining unassigned de novo sequences were searched on the online BLAST search engine MS-BLAST

(<http://genetics.bwh.harvard.edu/msblast/>)⁴². Peptide identification used a cutoff score of >20 (−10 lgP). Proteins were assigned as homologous when spots were located at the same position (mass and pI) on at least two 2D gels and the protein was identified from the peptide fragment in at least one of the gel spots. Functions were assigned according to the GO annotation within UniProt, where available. Spectra have been provided for proteins matched with a coverage of less than 2% and have been included in the supporting information file (Figure S1-S4).

Chemical Reagents

All chemicals used were of analytical grade and, unless otherwise stated, were sourced from Sigma Aldrich (NSW, Australia). All buffers were prepared using Milli-Q (18 MΩ/cm²) water.

Results

Peptidome Analysis

Initially, the venom collected from *P. clavata* obtained by electrical stimulation and venom gland dissection of colony 1 was subjected to MALDI-TOF MS analysis. Venom obtained by either method was dominated by peptides in the mass range 2600–3200 *m/z* (Figure 1Aa-Ba). It was also found that no peptides appeared to be present at masses greater than 3200 *m/z* and that manually dissected venom appeared to contain more peptides than electrically stimulated venom, particularly at masses <2100 *m/z*. Nevertheless, the most intense peptide signal in both electrically stimulated and manually dissected venoms was of mass 2786.3 and 2787.6 *m/z*, respectively (Figure 1 Ab-Bb).

Ants from colony 2 were also dissected and their venom glands pooled and analysed alongside those from colony 1 (Figure 1 Ca). The MALDI-TOF MS spectrum of the manually dissected colony 2 venom sample was similar to the one obtained from colony 1, particularly the dissected venom sample. However, colony 2 venom had differences in the relative intensities of the major peptides as well as the presence of numerous additional peptides below 1500 *m/z* (Figure 1Cb). Nevertheless, the most abundant peptide 2786.4 *m/z* matched that seen in colony 1 samples (±1 Da).

In order to reduce the complexity of the venoms, and therefore diminish the effects of ion suppression, all venoms were subsequently separated by C18 RP-HPLC. The HPLC chromatograms of all three venoms are shown in Figure 2A-C. It can be seen that the venoms obtained by venom gland dissection and electrical stimulation from the same colony (colony 1) have similar profiles, each with 39 fractions. However, while the venom elution profile from colony 2 has many similar peaks, overall it has a different profile with only 29 peaks and an additional dominant peak at 50 min (Figure 2C).

Although each RP-HPLC fraction was seemingly homogenous, mostly with clear sharp peaks, multiple peptides were observed to co-elute in the same LC fraction when further resolved by MALDI-TOF MS analysis. This varied from no peptides in some fractions up to 32 masses in fraction 39 of the dissected venom. Overall, each venom contained a large number of small mass peptides, with cumulative total counts of 115 masses for electrically stimulated venom from colony 1, 137 masses for manually dissected venom from colony 1 and 354 for manually dissected venom from colony 2. This highlights the overall complexity of *P. clavata* venom particularly from colony 2. Although both dissected venoms had a similar mass distribution, with 95% of peptides < 2.9 kDa (grey shaded areas in Figures 3B-C), the electrically stimulated venom from colony 1 contained a small number ($n = 15$; 13%) of higher mass peptides in the range 3–10 kDa that were not present in either of the dissected venoms from colony 1 or 2 (Figure 3A). Venom from colony 2 also had some similar, but not identical, high mass peptides, however they had a different RP-HPLC retention time compared to those from electrically stimulated venom from colony 1, likely representing unique peptides. This further highlights the complexity of venoms obtained from different colonies of *P. clavata*.

Although there were only subtle differences in the chromatograms and the peptide mass distributions of the three venoms, comparisons between the peptide mass lists from the three venoms revealed large differences between venoms from each of the two collection methods and also between different colonies. Figure 4 shows that venoms obtained by the two different collection methods from ants of colony 1 had only 22 common masses between them (Figure 4C-D). This means that 81% and 84% are peptides unique to electrically stimulated and manually dissected venoms, respectively, despite the fact that the ants belong to the same colony.

Proteome analysis

2D-PAGE analysis was performed in triplicate for each of the three samples, with representative gels shown in Figure 5. Overall the resultant gels look similar to one another, however when comparing the samples from the same colony (panels A and B of Figure 5), 24 spots were common (by mass and *pI*) to both the electrically stimulated venom (total of 53 spots; Figure 5A) and the manually dissected venom (total of 38 spots; Figure 5B) gels. Interestingly, the sample from colony 2 (Figure 5C) contained no unique spots that were not present in either of the other two samples, and was not subjected to further analysis.

In order to identify whether the different collection methods resulted in different venom protein profiles, nanoLC-ESI-QTOF MS/MS analysis was performed on the 2D gel spots from *P. clavata* venom obtained from colony 1 by the different extraction techniques. Due to the absence of any specific proteomic or transcriptomic data for *P. clavata*, several different databases were searched in order to determine the highest homology matches. Two search engines were employed, Mascot and Peaks, while MS Blast was used to determine proteins with high homology to peptide sequences determined by de novo sequencing of MS/MS spectra. An extensive list of protein matches from each spot is included as Supplementary Tables S1 and S2. A total of 73 and 75 proteins were identified from venom obtained by electrical stimulation and manual dissection, respectively (Figure 6A), of which 54 identified proteins were common to both venoms (Figure 6B). This showed that although there were proteins shared by the two collection methods (Table S3), there still were identified proteins unique to each method: 21 for electrically stimulated venom and 19 for manually dissected venom.

In the 2D gels (Figure 5) it can be seen that the most abundant proteins in all three gels was a train of spots (#12, #40–42, and #72) at ca. 18–21 kDa with an acidic *pI* of ca. 4.5–5.5. These spots were all identified as isoforms of the enzyme phospholipase A₂ (PLA₂) in all three venom samples. PLA₂ proteins have been identified in many other hymenopteran venoms^{3, 43-46}. The high abundance of these proteins, particularly in manually dissected venom from colony 1 (Figure 5B), and the necessity not to overdevelop the gel may have resulted in less abundant proteins being below the limit of detection, particularly with Figure 5B.

Other proteins that were common to all three venoms were spots #5, #25 and #26. MS/MS analysis of spot #5, located at ca. 51 kDa, with a pI of ca. 5–6.5, revealed a match to the plasma glutamate carboxypeptidase, a secreted protein involved in peptide metabolism, in both spots. This protein has a reported mass of 53 kDa and pI of 5.87 consistent with the spot's position. Spots #25 and #26 (ca. 25 kDa, pI 6.5) both matched to the protein gamma-interferon-inducible lysosomal thiol reductase, a 24 kDa protein with a pI of 6.5, involved in endosomal disulfide reduction.

As expected, Figure 6 shows that structural proteins are more prominent in manually dissected venom rather than electrically stimulated venom. For example, actin appeared at several spots in the manually dissected venom gel, including spots #5, #63 and #74 (Figure 5B) at a mass of 36–38 kDa and pI of ca. 5–7, but only in an isolated spot #8 from electrically stimulated venom (Figure 5A). Another example of a structural protein that was only found in manually dissected venom is flexible cuticle protein, a transmembrane protein, which was identified from spot #69 (Figure 5B) and was not seen in the electrically stimulated venom.

In order to simplify this data, all protein matches were classified into six different functional categories: toxins, regulation, metabolism, structural, transport and undetermined. Figure 6A shows that the two different collection methods result in venoms that contain functionally similar proteins. The main differences were seen in the structural and undetermined protein categories. The electrically stimulated venom had less structural and more undetermined proteins compared to the manually dissected venom (Figure 6A).

As seen in Fig. 6B and Table S3, each of the functional categories also had a high proportion of homologous proteins. For example, a regulatory protein identified from spot #19 in both gels was the 26S proteasome non-ATPase regulatory subunit 1 Tables S1 – S3. While an example of a toxin/allergen protein present in both venoms was PLA₂, which was seen in many spots such as in spots #12, #14, #42 and #43 (Tables S1 – S3). Another toxin identified was allergen sol i III⁴⁷, however it was only seen in spot #37 of the electrically stimulated venom.

Discussion

Venom collection is the rate-limiting step in venom studies of small animals and restricts further investigation for drug and bioinsecticide discovery, allergenicity studies and anti-venom development². The two main methods of collecting venoms are either electrostimulation or manual dissection of the venom gland⁴⁸, with the obvious disadvantage of the latter procedure being that the animal must be sacrificed. The present investigation used proteomic and peptidomic techniques to investigate the difference between these two collection methods and also identified differences in venom composition between different *P. clavata* ant colonies. While these two venom collection methods have previously been employed to investigate venom composition from other organisms such as bees³, cone snails¹³ and spiders²⁵, the present study represents the first systematic investigation of ant venoms obtained using these two methods. Both electrical stimulation^{5, 49} and manual dissection^{50, 51} collection methods for ant venoms have been used in separate studies; however, any differences between these two methods were not established. The present investigation represents the first in-depth systematic study of both the peptidome and proteome from a single species of ant and is the first comprehensive study of *P. clavata* venom, an ant that causes one of the most painful insect stings⁴⁸. Whilst there was no major disparity in the numbers of peptides and proteins in venoms collected using the two methods, closer investigation revealed that there were distinct differences in the masses of these peptides and the types of proteins.

Variations in Peptidome

The two collection methods were first compared based on the MALDI-TOF mass profiles of electrically stimulated versus manually dissected whole venom. *P. clavata* venom was found to be rich in peptides with no major differences between venom collection techniques. We therefore performed C18 RP-HPLC separation of the two venoms followed by offline analysis of the chromatographic fractions (LC-MALDI-TOF MS). This was to avoid the ion suppression effects known to occur in MALDI-TOF MS with complex venoms such as spiders, cone snails and both poneroid and formicoid ants^{9, 52}. The RP-HPLC chromatograms were not significantly different and the large number and mass distribution of the peptides below 4 kDa was similar to that reported for other poneroid ant venoms⁵¹⁻⁵⁴. This mass range of peptides is not unique to ant venoms, as cone snail venom contains mostly low molecular weight peptides in their venoms⁵⁵. Nevertheless, other venomous organisms such as spiders

and scorpions have peptides in the higher molecular weight range of approximately 3–10 kDa^{9, 56}. However, comparison of individual masses within ant venom LC fractions found that each collection method resulted in venoms with unique peptide masses. As expected, there were more peptides unique to manually dissected venom compared to electrically stimulated venom (42 vs. 31), probably reflecting peptides, or products of proteins, that are part of the venom gland itself or are part of the gland's machinery for toxin maturation and processing^{13, 25}. Other reasons for this high variability might be attributed to the pooled ants' having different diets⁵⁷ or being in different stages of development which may give rise to ontogenic variation as shown in several venomous animals such as wasps⁵⁸, spiders⁵⁹ and snakes⁶⁰. The process of electrical stimulation of the abdomen may not only release peptides from the venom gland, but also peptides and breakdown products of other glands. These may include antimicrobial and antifungal peptide contaminants from the digestive tract or hemolymph⁶¹ and peptides and proteins that are breakdown products involved in the biosynthesis of trail pheromones and other exocrine secretions.

Previous work on the ant *P. clavata* has been focussed on poneratoxin (PoTx), a neurotoxic peptide that was identified as the major component of the venom^{19, 21, 23}. Poneratoxin is a 25-residue peptide with an average mass of 2756.4 Da that has been investigated for its potential development as a bioinsecticide^{19, 20, 62}. More recently an investigation by Johnson et al. (2016) identified three new isoforms of poneratoxin with average masses of 2814.4 Da (glyceryl-PoTx; the presumed precursor of PoTx with an additional glycine residue at position 26), 2814.4 Da (A23E PoTx) and 2783.5 Da (D22N A23V PoTx) that were isolated from ants collected from different geographical locations. Post-translational modifications to peptides are not unique to *P. clavata* venom, but are common in many animal venoms such as other ants^{53, 63}, wasps⁶⁴, spiders⁶⁵ and especially marine cone snails⁵³. These modifications have been attributed to the high peptide counts seen in these venoms as well as the variations between species²⁹. In support of Johnson et al (2016) we also identified a peptide of mass ca. 2784.4 Da as the most dominant peptide in venoms obtained by the two collection methods from colony 1 and also from venom from colony 2 (see Figure 2 for elution times). Based on mass, we were only able to identify PoTx from venom obtained from colony 2. This further supports the idea that there are several poneratoxin homologs in *P. clavata* geographical variants and within venom from a single colony.

Variations in Proteome

The *P. clavata* proteome seems to be on a similar level of complexity, in terms of spot numbers, as other hymenopteran venoms^{3, 66} and other poneroid ants^{52, 67}. However, *P. clavata* venom is less complex, in terms of protein numbers and mass ranges, to that of other venomous organisms such as cone snails⁶⁸, snakes^{69, 70} and spiders which have up to 300 protein spots⁶⁹. Using a 2D-PAGE approach, there was also a large number of protein spots that were shared between the two collection methods, for example, spots #54 and #39 were resolved in the same position (mass and pI) in both electrically stimulated and manually dissected venoms. In contrast, there were several spots that were unique to each collection method: spot #29 in electrically stimulated venom and #14 manually dissected venom. While manually dissected venom was expected to have more proteins due to likely contamination of venom gland proteins^{3, 29}, a number of high abundance proteins may have obscured low abundance ones⁶⁶. This included a number of high intensity spots on the acidic end of the manually dissected venom gel at approximately 20 kDa, later identified as PLA₂ homologues.

nanoLC-ESI-QTOF MS-MS analysis of 2D gel spots identified several proteins within the venom using both collection methods. The most abundant proteins from electrically stimulated and manually dissected venoms were identified as PLA₂ homologues. This is a common venom toxin reported in wasp⁷¹, bee³, snake⁷⁴ and ant^{44, 52, 72} venom as well as being a major allergen in hymenopteran⁷³ venoms. Its activity involves disruption of phospholipid membranes leading to pore formation, cell lysis, cardiac dysfunction and ultimately death of the organism⁷¹. Other stinging hymenoptera have also been found to have high levels of PLA₂ that account for at least some their toxic activity⁷⁵. For example, 12% of the dry weight of bumblebee venom are PLA₂s⁷⁵. The lipase property of phospholipases may also facilitate further spreading of venom through the host tissue¹³. The allergen sol i III, originally identified in the venom of the ant *Solenopsis invicta*, was also present in *P. clavata* venom, and may contribute to the overall toxicity, particularly allergic responses⁴⁷ despite these have not been widely reported with *P. clavata* envenomation. As there were no other proteins with potential toxic activity identified, it appears that the toxic effects of *P. clavata* venom are the result of poneratoxin as suggested by Johnson¹⁶, perhaps in combination with PLA₂ isoforms. Nevertheless, the sting by *P. clavata* does not

just involve a very painful sting, but also other systemic effects such as haemolysis⁷⁶ and tonic-clonic seizures that have been observed in mice that ultimately lead to death at relatively low doses (6 mg/kg)⁷⁷. The venom components that cause these additional activities remain unidentified.

The 2D gels of *P. clavata* venom obtained in this study contained several spots that were identified as PLA₂ proteins, an observation reported for other venoms, including cone snails, snakes and wasps^{29, 73, 74}. Interestingly, the PLA₂ proteins have a range of different *pI* values and masses. Variance in the *pI* of PLA₂ homologues present in *P. clavata* venom can be explained by differences in the amino acid sequence of PLA₂ subunits resulting in a range of basic and acidic PLA₂ proteins, as previously described in hymenopterans and snakes^{73, 74}. The variability in mass can be explained by homology with multimers of PLA₂ subunits (often with different individual subunit masses) or monomer PLA₂ proteins, also with varying degrees of glycosylation on individual subunits⁷⁴. Despite the coverage scores of the matched proteins not being particularly high, it can be safely assumed that the protein matches are correct, as this enzyme is known to be highly conserved within *Hymenoptera*⁷³ and has also been reported in *P. clavata* using biochemical assays^{76, 77}.

Although peptides perform a significant role in venom functions, proteins can act as carriers, neurotoxins or derivative enzymes such as proteases to help in the maturation and function of the peptides¹³. These proteins may also have synergistic effects on the toxic components of venoms⁶⁵. Our study identified several manually dissected venom proteins that are located intracellularly, and we observed that electrically stimulated venom had fewer of these. In particular these were structural proteins such as actin and cellular transport proteins such as myosin. This has also been reported in previous work that compared these two techniques using other venoms such as that of bees and marine cone snails^{66, 78}. It has therefore been claimed that electrically stimulated venom is more representative of the injected venom compared to manually dissected venom³. The intracellular proteins are usually thought of as artefacts of dissection, however it has been proposed that they might be co-secreted into the venom gland along with a toxin^{29, 65}. In support, we saw some structural proteins in electrically stimulated venom as well.

The relative proportion of proteins allocated to each category in this study was not markedly different to those of other venoms, with the majority characterised as intracellular proteins^{3, 29}. There was also a large number of proteins in the unknown category, a recurring issue in most venomomics studies. This is due to the lack of genomic and transcriptomic bioinformatics data available for a number of venoms, especially those of ants, as well as the added problem of low abundance 'masking' due to high abundance proteins^{25, 65, 79}.

Inter-colony Variations

There were several differences between the venom from ants of colony 1 to those of colony 2 in both the peptide and protein numbers and protein composition. This has been previously observed with other ant species such as *Odontomachus haematodus*⁸⁰, *Dinoponera quadriceps*⁵³ and other venomous organisms including marine cone snails¹¹ and wasps⁶⁴. The reason for these marked differences has been attributed to different factors such as age, size, differential gene expression, altered post-translational modifications and, in the case of ants, genetic polymorphisms that result from the queens mating with different males during their lifetime^{64, 81}. It has also been previously shown that although the organisms are from the same region, as was the case with these colonies, slight environmental differences between the two colonies may explain the differences seen^{53, 64}. For example, for a predatory ant like *P. clavata*, prey diversity and abundance, as well as other factors such as peptide composition during venom regeneration and the overall health of individual ants may affect peptide levels and expression⁵³.

Concluding Remarks

The present study has shown that venom obtained by manual venom gland dissection reveals a broader number of peptides and proteins than electrically stimulated *P. clavata* venom. From the 2D-PAGE analysis, approximately 72–74% of the proteins are shared by the two collection methods. It was found that the manually dissected venom contained a number of additional regulatory proteins, while the electrically stimulated venom had a number of undetermined proteins. However, there is no evidence suggesting that these additional undetermined and regulatory proteins are toxic. Unexpectedly, the overlap in peptide mass matches (approximately 16–19%) was far less than what was observed from

protein matches. Although extracting venom via electrical stimulation is the preferred method of collection, as it is not destructive to the ants and contains all the major protein components, this work collected electrically stimulated venom by individually milking ants which was not an efficient method, and future work should utilise a mass milking technique such as that described by Eskridge et al. (1981)⁸². It must be noted that the peptide composition differs markedly to that of manually dissected venom and both methods may need to be used to obtain a more accurate representation of venom contents. This venom complexity is further increased when venoms from different colonies are compared. This highlights the diversity of ant venom proteins and peptides that can be characterised between colonies, and by using different collection techniques, for the discovery of potential therapeutic or insecticidal leads.

Supporting Information

The following files are available free of charge at ACS website <http://pubs.acs.org>:

- **Supplementary Table S1:** Protein assignment of *P. clavata* dissected venom 2D-PAGE spots identified by nanoLC-ESI-QTOF MS/MS
- **Supplementary Table S2:** Protein assignment of *P. clavata* electrically stimulated venom 2D-PAGE spots identified by nanoLC-ESI-QTOF MS/MS
- **Supplementary Table S3:** *P. clavata* proteins identified from 2D-PAGE spots of electrically stimulated and manually dissected venom. Table shows proteins identified by each method as well as proteins unique to each collection method
- **Supplementary Figure S1:** Annotated MS spectra of peptides from manually dissected *P. clavata* 2D gel spot 22 used to match against hymenopteran proteins.
- **Supplementary Figure S2:** Annotated MS spectra of peptides from manually dissected *P. clavata* 2D gel spot 58 used to match against hymenopteran proteins.
- **Supplementary Figure S3:** Annotated MS spectra of peptides from electrically stimulated *P. clavata* 2D gel spot 26 used to match against hymenopteran proteins.
- **Supplementary Figure S4:** Annotated MS spectra of peptides from electrically stimulated *P. clavata* 2D gel spot 45 used to match against hymenopteran proteins.

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Abbreviations

ACN, acetonitrile; C7BzO, 3-(4-heptyl)phenyl-3-hydroxypropyl) dimethylammonio propane sulfonate; 1,5-DAN, 1,5-diaminonaphthalene; CHCA, α -cyano-4-hydroxycinnamic acid; FA, ferulic acid; HGD, Hymenopteran Genome Database; IEF, isoelectric focussing; ionic CHCA, N-tert-butyl-N-isopropyl-N-methylammonium α -cyano-4-hydroxycinnamate; IPG, immobilized pH gradient, LC-ESI-QTOF MS, liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry; MALDI-TOF MS, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; PLA2, phospholipase A2; RP-HPLC, reverse-phase high pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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Figure Legends

Figure 1. Representative MALDI-TOF MS spectra of *P. clavata* whole venom. Venom was obtained by electrical stimulation (Panel A) and manual dissection (Panels B and C) from colony 1 (panels A and B) and colony 2 (panel C). Initial screening was between 1000–10,000 m/z (left-hand panels Aa-Ca). Right-hand panels show mass spectra between 1000–3500 m/z where peptide abundance was the highest (panels Ab-Cb).

Figure 2. RP-HPLC chromatograms of *P. clavata* venom glands extracted by electrical stimulation or venom gland dissection. Venoms were separated by analytical C18 RP-HPLC using an ACN/0.085% TFA gradient of 1%/min (grey dotted line) at 1 ml/min. Panels show the chromatographic elution profile of *P. clavata* venoms from colony 1 obtained by (A) electrical stimulation and (B) manual dissection. Panel C shows profile of venom from colony 2 obtained by manual dissection. Chromatographic peaks were monitored at 215 and 280 nm (only absorbance at 215 nm is shown for clarity). Peaks in which poneratoxin was found are labelled as “Potx”.

Figure 3. LC-MALDI-TOF mass distribution and total peptide count of *P. clavata* venom obtained using the different venom collection methods. (A-C) Histograms show the abundance of peptide masses in each venom sorted into 100 Da mass bins (columns; left-hand ordinate axis) and the cumulative total of peptide masses in each venom (circles; right-hand ordinate axis). Total peptide counts are indicated in the upper right-hand corner of each panel. (A) *P. clavata* venom obtained by electrical stimulation of ants from colony 1, (B) manual dissection of ants from colony 1 and (C) manually dissection of ants from colony 2. Shaded areas represent 5–95% of the total number of peptides.

Figure 4 2D landscapes of *P. clavata* venom peptides from colony 1 determined using MALDI-TOF MS. Panels show venom peptides collected by (A) electrical stimulation, (B) manual dissection, and (C) peptides common to both collection methods based on a mass tolerance of ± 1 Da. Grey shaded areas represent 5–95% of peptide masses. Inset in panel C shows an area-proportional Euler plot depicting variation in numbers of shared and distinct peptides. Potx is represented by red circles. DV, manually dissected venom; ESV, electrically stimulated venom. (D) Mass list for peptides common to manually dissected and electrically stimulated venom. Potx is indicated by a red shaded box.

Figure 5. 2D SDS-PAGE of reduced and alkylated *P. clavata* venoms. Venoms were extracted by (A) electrical stimulation of ants from colony 1, (B) dissection of the venom glands of ants from colony 1, and (C) dissection of the venom glands of ants from colony 2. Representative gels from triplicates of each sample are shown. A total of (A) 53 spots, (B) 38 spots (circled) were excised for identification by nanoLC-ESI-QTOF MS/MS. In panel C 57 spots (circled) were observed, but no spots were unique from panels A and B. Circles/ovals represent shared proteins between A, B and C (yellow), A and C (red) and B and C (green). Black circles/ovals represent unique proteins not detected in the other gels.

Figure 6. Functions of identified *P. clavata* venom proteins from colony 1. A Pie graph depicting classification of venom proteins identified from 2D-PAGE spots using nanoLC-ESI-QTOF MS/MS. Panels show venom proteins obtained by electrical stimulation (left-hand panel) and manually dissected venom (right-hand panel). B Area-proportional Euler plots depicting numbers of homologous and distinct venom proteins obtained by electrical stimulation (ESV; cyan circles) and manual dissection (DV; brown circles).

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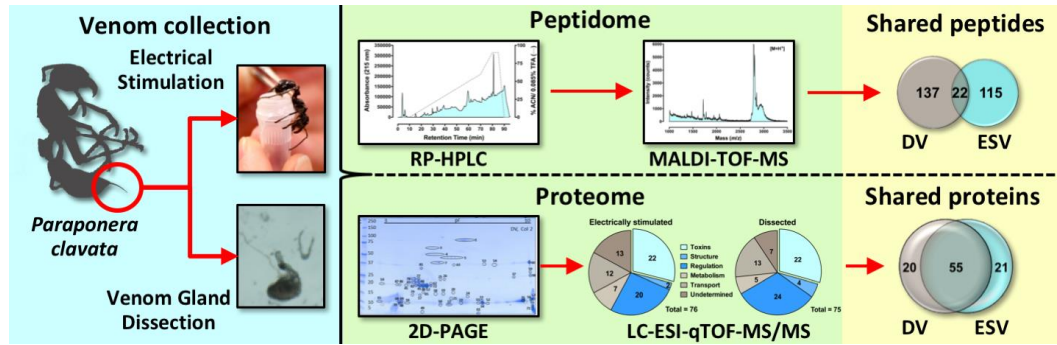


Photo courtesy of Axel Touchard