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Characterization of the Venom Proteome for the Wandering Spider, *Ctenus hibernalis* (Aranea: Ctenidae)

Jeffrey Cole[1], Patrick A Buszka[1], James A Mobley[2] and Robert A Hataway[1]*

[1]*Department of Biological and Environmental Science, Samford University, Birmingham, AL 35229-2234, USA*
[2]*Department of Surgery, University of Alabama-Birmingham, Birmingham, AL 35294-0113, USA*

Abstract

Spider venom is a rich multicomponent mixture of neurotoxic polypeptides. The venom of a small percentage of the currently classified spiders has been categorized. In order to determine what venom proteins are expressed in our species, the wandering spider *Ctenus hibernalis*, we constructed a comprehensive proteome derived from a crude venom extract using a GeLC approach that required a one dimensional denatured gel electrophoresis separation combined with enzymatic digestion of the entire lane cut into many molecular weight fractions followed by LC-ESI-MS2. In this way, we identified 1,182 proteins with >99% confidence that closely matched sequences derived from the combined genomes taken from several similar species of spiders. Our results suggest that the venom proteins of *C. hibernalis* contain several proteins with conserved sequences similar to other species. Going forward, with next generation sequencing (NSG), combined with extended annotations will be used to construct a more complete genoproteomic database. Therefore, it is expected that with further studies like this, there will be a continued and growing understand of the genoproteomic makeup of the venom for many species derived from insects, plants, and animals. We believe that as a whole these approaches will lead to a much better understanding of the biology behind venoms of all types, as well as ways to treat exposed patients while also expanding upon and taking advantage of the various positive sides of venomous toxins.

Keywords: Venom; Spider; Proteome; Ctenidae; Ctenus

Introduction

Spider venoms are a multicomponent mixture of polypeptides that contain a diverse array of structure and function that is used for both the immobilization of prey as well as a defense mechanism [1-4]. To date, the venom composition of less than 100 of the nearly 40,000 characterized species of spiders has been investigated [5]. Although certain venom protein families are highly conserved across spider taxa [6], there are several instances of novel taxa-specific venom proteins, such as latrotoxins in *Latrodectus,* Sphyngomyelinase D in *Loxosceles*, and μ-ctenitoxin-Pn1a in *Phoneutria* [7-9]. Spider venom has been shown to have several therapeutic applications due to the vast array of biological functionality such as neurotoxic, antimicrobial, antiparasitic, cytolytic, hemolytic, and antiarrhythmic activities [10]; it is thus likely that undiscovered peptides of novel importance are likely to be found in previously unexplored venoms.

Spiders in the Ctenidae family, a group containing nearly 500 species in 42 genera that range mostly in tropical terrains, is home to the most venomous spider in the world *Phoneutria nigriventer* [11], and a nonlethal spider that has become the model species for arachnological studies on evolution and development *Cuppienius salei* [12]; both of which are South American spiders whose venom has been highly studied [13,14]. In the U.S., *Ctenus hibernalis* is one of only 7 representative species of Ctenidae spiders and it has primarily been collected in Alabama [15], but little to no information is available about its ecology or physiology*,* nor is there anything known about its divergence from its tropical counterparts in relation to its venom. The aim of this study is to utilize proteomics techniques in order to characterize the venom proteome of *C. hibernalis* and to determine what similarities exist between its venom composition and other spider taxa as well as its tropical counterparts.

Methods

Spider collection

Individuals were hand collected at night, the time when they are most active, using spotlight techniques due to the reflective tapetum within their eyes [16]. Collection was done within the Homewood Forest Preserve in Homewood, AL in September 2015. Only adult females, collected within the same week, were included to limit confounding variables such as ontological differences in venom composition that may occur over time and between sexes as has occurred in other species [17-19].

Venom collection

Prior to venom collection, individuals were anesthetized with CO_2 as previously described [20]. Venom was collected using electrostimulation with 7V of AC current, similar to previous studies [21-24]. Anesthetized individuals were placed on clamped forceps attached to an electrode. One prong of the forceps was wrapped in nonconductive insulating tape to create a point of contact for the spider that would retard current, while the other prong of the forceps was wrapped with a cotton thread and soaked in saline to create a point of contact with the spider to promote electrical conductivity. A capillary tube was then placed over the fang in order to collect the venom. Finally, the second electrode was touched to the base of the chelicerae in order to complete the circuit and allow the muscles around the venom gland to contract and eject venom into the capillary tube. Venom was pooled from 21 individuals and then stored at -80°C prior to analysis according to previously reported methodology [25,26].

*Corresponding author: Robert A Hataway, Department of Biological and Environmental Science, Samford University, Birmingham, AL 35229-2234, USA, Tel: 205-726-4190; E-mail: rahatawa@samford.edu

Sample preparation and data acquisition

The venom protein isolates were quantified against an 8-point standard curve run in triplicate using a BCA Protein Quantification Kit (Invitrogen), 50 μg was diluted in LDS PAGE buffer (Invitrogen) containing reducing agent and separated on a 4-2% SDS Bis-Tris gel (Invitrogen). The gel was stained overnight with colloidal blue (Invitrogen), and destained prior to visualization. The entire lane was cut into 19 molecular weight (MW) fractions. Gel slices were reduced, carbidomethylated, dehydrated, and digested with Trypsin Gold (Promega) as per manufacturers' instructions. Peptide digests were analyzed in duplicate using an LTQ XL ion trap mass spectrometer equipped with a nano-electrospray source, and a Surveyor plus binary high-pressure liquid chromatography (HPLC) pump (Thermo Scientific, San Jose CA) using a split flow configuration. Separations were carried out using a 75 μm × 13 cm pulled tip C-18 column (Jupiter C-18 300 A, 5 μm, Phenomenex).

Data analysis

The data was searched using SEQUEST (v.27 rev12, .dta files). Searches were performed using all published Araneae venom peptide sequences in Uniprot that also contained common contaminants such as porcine digestion enzymes and human keratins.

Identified peptides were filtered, grouped, and quantified using Scaffold (Proteome Software, Portland Oregon). Only peptides with charge state of ≥ 2+, a minimum peptide length of 6 amino acids, were accepted for this analysis, in addition to proteins containing ≥ 2 peptides and a final false discovery rate of <1%. Relative quantification was performed via spectral counting, and spectral count abundances were normalized across the entire set. Toxin groups, delineated by taxonomic family, and molecular targets were identified according to activity prefix from King et al. [27,28].

Results

A total of 21 female *C. hibernalis* were collected for venom sampling. Pooled venom from the 21 individuals provided a total of 20 μL with a protein content of 53.8 μg/μL as determined by BCA assay. The proteins were separated into 19 MW fractions in the 1D gel ranging from 3-188 kDa (Figure 1) for downstream analysis by LCMS2. From these data, 1,182 proteins matched the published spider venom sequences with >99% confidence. A match required at least 2 peptides uniquely mapped to the protein. These matches ranged from 93 species in 27 spider families (Figure 2a). Of those 1,182 proteins, 86 were found in other Ctenidae spiders, and 335 of these matches had an attributed molecular activity prefix (Figure 2b). There were strong matches with cytolytic proteins as well as proteins involved in channel inhibition (Ca^{2+} Na^+ and K^+) (Table 1, Supplementary Material).

Discussion

This study marks not only the first attempt to characterize the venom of *Ctenus hibernalis,* but also the venom composition of a U.S. native Ctenidae. We detected over a thousand unique proteins homologous with venom proteins across several spider taxa, which indicates the venom proteins are highly conserved. Although venom proteins in evolutionarily young clades of venomous animals such as snakes that diverged 30-50 million years ago (MYA) typically undergo positive selection due to a predatory arms race [29,30], it has been discovered that certain venom protein families in the ancient clade of spiders that diverged 416-359 MYA have been optimized for their predatory purposes and undergo purifying selection to conserve venom protein functionality [6,31].

The venom of *C. hibernalis* has been determined to contain a diverse array of biological functionality. This variable composition is signature of predatory venoms found throughout several taxa of venomous predators [32]. The complex nature of the venom provides further insight into the venom strategy of *C. hibernalis.* The array of channel inhibitors detected in the venom of *C. hibernalis* indicates a strong potential for neurotoxicity as seen in previous studies [33-35]. Co-injection of these several ion channel inhibitors is likely to have synergistic effects similar to what has been discovered in the agotoxins of the American funnel-web spider *Agelenopsos aperta*. A particularly surprising match was with that of μ-ctenitoxin-Pn1a found in *P. nigriventer* that has been determined to be the lethal component [7], so it is unexpected to be detected in the venom of *C. hibernalis* venom, which is not lethal to humans. Future expression level investigations and structural comparisons will be necessary to determine the use of μ-ctenitoxin-Pn1a in *C. hibernalis*. Amongst the cytolitic matches were several antimicrobial peptides. This suggests that *C. hibernalis* may be an ideal source for therapeutic antimicrobial peptides.
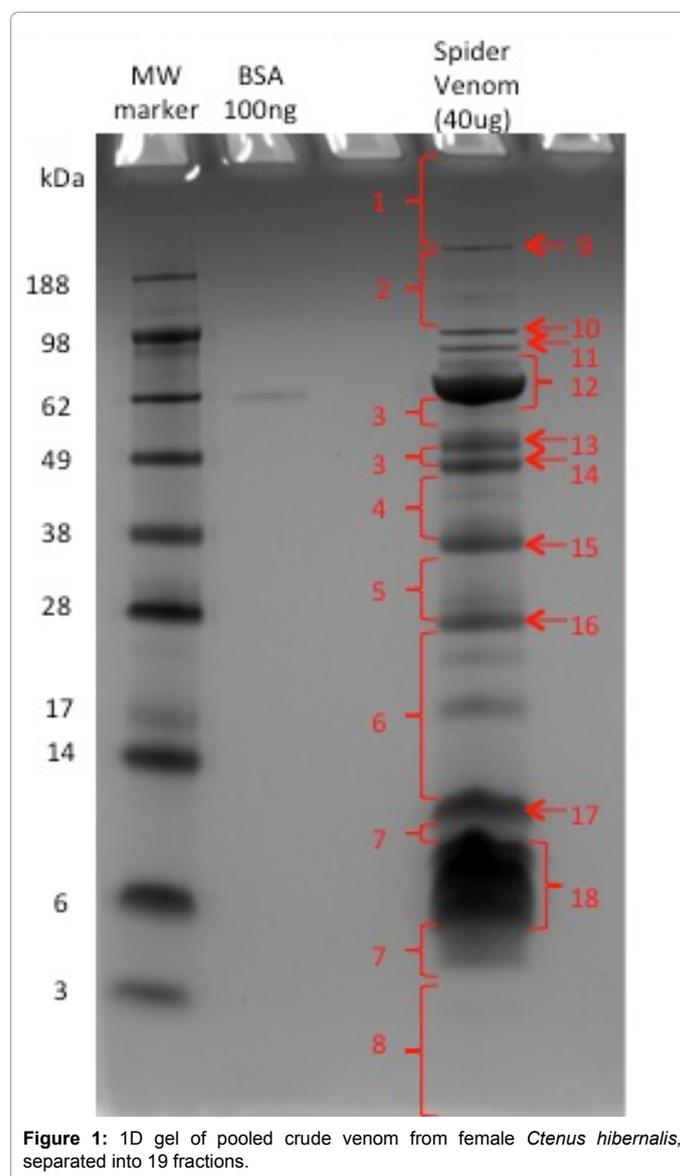

Figure 1: 1D gel of pooled crude venom from female *Ctenus hibernalis,* separated into 19 fractions.
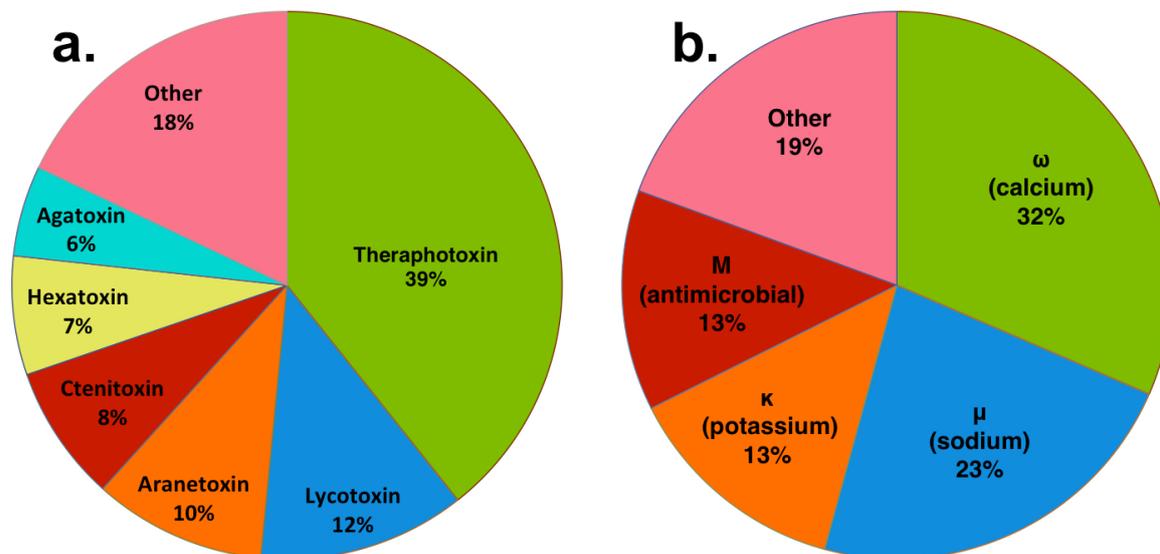
Figure 2: a) Distribution of homologous spider toxin families of detected proteins in the venom of *Ctenus hibernalis*. Other spider venom families such as pisautoxin, barytoxin, zodatoxin, oxotoxin, miturgitoxin, filistitoxin segestritoxin, latrotoxin, plectotoxin, cyrtautoxin, sicaritoxin, sparatoxin, amaurobitoxin, diguetoxin, nemetoxin, thomitoxin were also detected and grouped into the the "other" section. b) Distribution of prefix activities from the 335 matches that had a characterized activity prefix from. The preifix ω (omega) inhibits voltage-gated calcium (Ca$_v$) channels, μ (mu) inhibits voltage-activated sodium (Na$_v$) channels, κ (kappa) inhibits voltage-activated potassium (K$_v$) channels, M (Mu) indicates haemolytic cytolytic or antimicrobial. Lumped into the "other" category are matches with the prefix δ (delta) delays inactivation of voltage-activated Na$_v$ channels, β (beta) shifts voltage-dependence of Na$_v$ channel activation α (alpha) targets acetylcholine receptor, γ (gamma) targets HCN nonspecific cation channels, and τ (tau) targets transient receptor potential (TRP) channel.

Accesion# (Uniref 100)	Homologous toxins name	Species	Peptide count	Molecular target	Coverage (%)
A9QQ26	CRISP-1-Lycosa	*Lycosa singoriensis*	47	Cysteine-rich secretory protein	97.9
B3EWF4	U2-zodatoxin-Lt2a	*Lachesana tarabaevi*	38	Insecticidal	100
D5GSJ8	Δ-miturgitoxin-Cp1a	*Cheiracanthium punctorium*	44	Cytolitic	72.7
G4V4G1	IGFBP_rP1-1-Cupiennius	*Cupiennius salei*	36	Insulin-like growth factor	93.1
GB000059	ω-theraphotoxin-Hs1a_2	*Haplopelma schmidti*	13	Calcium channel	100
GB000132	ω-filistatoxin-Kh2b	*Kukulcania hibernalis*	15	Calcium channel	98.6
O76199	δ-ctenitoxin-Pn2c	*Phoneutria nigriventer*	11	Sodium channel	100
O76200	κ-ctenitoxin-Pn1a	*Phoneutria nigriventer*	18	Potassium channel	55.4
O76201	ω-ctenitoxin-Pn1a	*Phoneutria nigriventer*	24	Calcium channels	95.1
P0C2U6	M-lycotoxin-Ls3b	*Lycosa singoriensis*	4	Antimicrobial peptide	100
P0DM68	U4-theraphotoxin-Spl1a	*Selenotholus plumipes*	19	Insecticidal	100
P17727	μ-ctenitoxin-Pn1a	*Phoneutria nigriventer*	10	Sodium channel	70.5
P29425	δ-ctenitoxin-Pn2a	*Phoneutria nigriventer*	7	Sodium channel	100
P36984	U1-plectoxin-Pt1b	*Plectreurys tristis*	5	Insecticidal	100
P49267	U1-cyrtautoxin-As1a	*Apomastus schlingeri*	11	Insecticidal	100
P58425	κ-sparatoxin-Hv1a	*Heteropoda venatoria*	3	Potassium channel	100
P58605	ω-segestritoxin-Sf1a	*Segestria florentina*	17	Calcium channel	100
P59367	γ-ctenitoxin-Pn1a	*Phoneutria nigriventer*	4	NMDA-glutamate receptor	67.9
P60978	U1-nemetoxin-Csp1c	*Calisoga sp.*	5	Insecticidal	100
P81694	ω-ctenitoxin-Cs1a 292	*Cupiennius salei*	16	Calcium channel	95.9
P83256	δ-Amaurobitoxin-Pl1a	*Pireneitega luctuosa*	4	Insecticidal	100
P83620	M-ctenitoxin-Cs1b	*Cupiennius salei*	12	Antimicrobial peptide	100
P84033	U21-ctenitoxin-Pn1a	*Phoneutria nigriventer*	10	Protease	100
P85505	μ-thomitoxin-Hme1a	*Heriaeus melloteei*	6	Sodium channel	100
Q25338	δ-Latroinsectotoxin-Lt1a	*Latrodectus tredecimguttatus*	110	Insecticidal	63.5
Q8MTX1	U3-aranetoxin-Ce1a	*Caerostris extrusa*	24	Unknown	100
W4VSH9	U19-barytoxin-Tl1a	*Trittame loki*	28	Serene protease	100

Table 1: Partial list of detected proteins in the venom of *Ctenus hibernalis* highlighting several species and molecular functionality, sorted by Uniref accession number. An exhaustive list of detected proteins has been included as supplementary material.

We were only able to characterize the venom proteins that had significant matches within the database; there are still several peptides to be characterized that are entirely unique to *C. hibernalis*. It is evident that the knowledge base of venom peptide sequences in Ctenidae spiders is limited, which merits further investigation. Future work is necessary to determine the sequences of peptides found in the venom that are not found in the database by generating transcriptomic data from the spider's venom gland. This ongoing work will allow for orthogonal validation and entire peptide sequences for all expressed venom proteins, rather than just the partial sequences generated from this study, which will also aid in determining the gene ontology distribution found in the venom proteins. Additionally this information will allow for future work quantifying expression levels in this species and its relatives using mRNA based methodologies. Further work is also necessary to expand the taxa that we investigated, as well as expanding to the population level using both proteomic and transcriptomic techniques in tandem to generate comprehensive venomic information of several species as well as individuals within a population. This will help generate a better understanding of the molecular evolution of venom proteins in these animals.

Acknowledgements

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