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Proteomic Analysis Provides Insights on Venom Processing in *Conus textile*

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Abstract

Conus species of marine snails deliver a potent collection of toxins from the venom duct via a long proboscis attached to a harpoon tooth. Conotoxins are known to possess powerful neurological effects and some have been developed for therapeutic uses. Using mass-spectrometry based proteomics, qualitative and quantitative differences in conotoxin components were found in the proximal, central and distal sections of the *C. textile* venom duct suggesting specialization of duct sections for biosynthesis of particular conotoxins. Reversed phase HPLC followed by Orbitrap mass spectrometry and data analysis using SEQUEST and ProLuCID identified 31 conotoxin sequences and 25 post-translational modification (PTM) variants with King-Kong 2 peptide being the most abundant. Several previously unreported variants of known conopeptides and were found and this is the first time that HyVal is reported for a disulfide rich *Conus* peptide. Differential expression along the venom duct, production of PTM variants, alternative proteolytic cleavage sites, and venom processing enroute to the proboscis all appear to contribute to enriching the combinatorial pool of conopeptides and producing the appropriate formulation for a particular hunting situation. The complimentary tools of mass spectrometry-based proteomics and molecular biology can greatly accelerate the discovery of *Conus* peptides and provide insights on envenomation and other biological strategies of cone snails.

Keywords

conotoxin; conopeptides; proteomics; differential expression; post-translational modification; Orbitrap

INTRODUCTION

Interests in the biochemistry of animal venoms started decades ago. [1-3] Over the years, the venom components have been isolated, characterized and used as biochemical tools to probe the mammalian neuromuscular system. One that has caught the attention of biologists, neuroscientists and the pharmaceutical industry is the venom of *Conus* species of marine gastropods, each of which contain a potent cocktail of 100-200 neuroactive peptides.[4-7] It is estimated that 50,000 – 100,000 different peptides exist in the venom of the 500-700 species

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of the genus *Conus*. Although only a few percent of *Conus* peptides have been sequenced and studied, they are already well established and widely used as molecular tools in neuroscience with several in clinical trials and one has been approved as a drug against pain.[8]

Sequencing of cDNA from the *Conus textile* venom duct[9] revealed that each peptide is encoded by a distinct mRNA with a prepropeptide organization. Subsequent studies on many conotoxins from different species indicated that the prepropeptides of conotoxins belonging to the same superfamily have a highly conserved signal sequence (pre region), a relatively conserved pro-region, and a highly variable C-terminal toxin-encoding region. Within the hypervariable toxin region are highly conserved cysteine residues corresponding to disulfide patterns characteristic of particular gene superfamilies, such as Osuperfamily, M-superfamily, A-superfamily.[10-13] A superfamily may consist of several families, each family targeting a specific ion channel and/or receptor.

Initial sequencing efforts involved chromatographic methods for the isolation of individual *Conus* peptides and Edman degradation for amino acid sequencing of conotoxins. Advances in molecular biology, particularly the expressed sequence tag strategy coupled with automated nucleic acid sequencing have facilitated the use of cDNA libraries of *Conus* venom ducts in determining conopeptide sequences.[14-15]However, many of the peptides predicted from conotoxin gene transcripts have not yet been isolated and their degrees of post-translational modification remain uncertain.

Mass spectrometry-based sequencing offers a rapid approach for the elucidation of conotoxin structures. It requires relatively small amount of sample compared to chemical sequencing via Edman degradation and more importantly, samples usually need not be in a purified form. A recent development in mass spectrometry, Makarov's Orbitrap is capable of detecting ions with high resolution by their motion in the spindle-shaped electrode[16] and its high mass accuracy and tandem mass spectrometry capability can provide unambiguous identification of peptides and proteins.[17-18] High resolution and high accuracy mass measurement should enable more rapid exploration of the *Conus* venom peptide library and at the same time identify post-translational modifications.

Proteomic profiling of venom from different portions of the *C. textile* duct may provide insights on the overall envenomation strategy used by *Conus* species. For example, a puzzling observation of Endean and co-workers[1,19] in *Conus* species is the higher potency of venom from the posterior (proximal) end of the duct that is attached to the venom bulb compared to the venom extracted from the anterior (distal) portion near the proboscis. More recently, Marshall et al. (2002) observed a variation in epithelial composition in the proximal and distal portions of the duct and suggested that this may be responsible for the variation in conotoxin expression along the venom duct.[20]

To address the localization issue of conotoxin expression in the venom duct, venom samples were obtained from the proximal, central and distal portions of the tubular duct of *Conus textile* and analyzed by using a LTQ-Orbitrap tandem mass spectrometer to identify the toxins present. Database search was performed using SEQUEST[21] for unmodified peptides and the ProLuCID search engine[22] for identification of post-translationally modified peptides. The post-search summary program DTAselect was used to assemble the results using a reverse database approach.[23] Conotoxins identified in the three duct sections were quantified using spectral counting[24] and the number of spectra matching each peptide/protein was taken as a measure of relative concentrations.[24-25]

In view of previous observations on *Conus* biology, the proteomic data on conotoxin distribution along the duct were examined to gain insights on the envenomation process. The

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different levels of post-translational modification found in many peptides indicate a much greater diversity of peptides in *Conus* venom than can be gleaned from the available molecular biology data.

MATERIALS AND METHODS

Sample Preparation and Venom Extraction

Cone snails were gathered from around the island of Marinduque, kept in bamboo cages in the sea before they were delivered to the laboratory, and frozen. A single specimen of *C. textile* (8 cm in length) was dissected and its venom duct cut into three equal sections. (Gloves and/or tongs were used when handling specimens, venom and purified components.) Venom was obtained by rolling each part of the duct until all the contents were released. The fresh venom (~100mg) from each section of the venom duct was shaken in separate tubes with 400 μL of cold distilled water for 5 minutes then 800 μL of 40% acetonitrile (ACN) was added and mixed for 20 minutes before adding 1.8 mL of pure ACN. The suspension was sonicated for 30 minutes in ice water after each successive addition of ACN. Finally, the pellet, which is composed of insoluble granules and cellular debris from the venom duct, was separated by centrifugation (10,000 × g) for 20 minutes. The supernatant was collected and stored at −80°C.

Liquid Chromatography and Mass Spectrometry

An aliquot of the extracted venom was denatured with urea, reduced with TCEP and alkylated with iodoacetamide using a procedure slightly modified from Washburn et al. (2001).[26] The reaction mixture was aliquoted and stored at −80 °C prior to use. An aliquot of venom was pressure-loaded onto a 100-μm i.d capillary with a 5-μm pulled tip packed with 10 cm 5-μm Aqua C18 material (Phenomenex, Ventura, CA). The column was washed with buffer containing 95% water, 5% ACN, and 0.1% formic acid for 30 min to remove salt. After desalting, the capillary column was placed in line with the mass spectrometer and analyzed as described below. An Eksigent quaternary HPLC was used to deliver buffer solutions of 5% ACN/0.1% formic acid (buffer A), and 80% ACN/0.1% formic acid (buffer B). Elution was done with a 180-min gradient from 0-100% buffer B.

Peptides eluting from the microcapillary column were electrosprayed directly into an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with the application of a distal 2.5 kV spray voltage. A cycle of one full scan of the mass range (MS) (400-2000 m/z, resolution of 60,000) followed by three data-dependent tandem mass spectra (MS/MS) (resolution of 30,000) with normalized collision energy (setting of 35%) in the Orbitrap was repeated continuously throughout each step of the multidimensional separation. Application of mass spectrometer scan functions and HPLC solvent gradients were controlled through the XCalibur data system.

Data analysis

The SEQUEST algorithm[21] was used for MS/MS database search of unmodified peptides, while the ProLuCID algorithm[22] was used for database search of post-translationally modified peptides. Tandem mass spectra were searched against a protein database comprised of the following: all ConoServer proteins (2368 entries downloaded from http://research1t.imb.uq.edu.au/conoserver/ on Oct 17, 2008); cone snail related proteins from NCBI (5381 entries, downloaded from http://www.ncbi.nlm.nih.gov/ on Oct 17, 2008); common contaminating proteins such as keratins; and EBI International Protein Index (IPI) human protein database version 3.48 (Downloaded September 1, 2008) concatenated to a decoy database in which the sequence for each entry in the original database was reversed.[23]

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The human protein database was included to serve as a background database to increase the probability of random matches.

SEQUEST MS/MS database search parameters were as follows: no tryptic terminus was required for candidate peptides; mass tolerance of the precursor ions was +/− 50 ppm. No post-translation modification was considered for the first round of database search. SEQUEST results were assembled and filtered with the DTASelect (version 2.0)[27,28] program. The DTASelect 2.0 program uses a linear discriminant analysis to dynamically set XCorr and DeltaCN thresholds for the entire dataset to achieve a user-specified false positive rate (0% in this study) assessed by the reverse hits. Proteins identified by the same peptide sets were clustered together by DTASelect 2.0. The program assembles identified peptides into proteins and protein groups by using a parsimony principle in which the minimum set of proteins accounts for all the observed peptides.

After SEQUEST search and DTASelect filtering, identified MS/MS spectra were removed. The remaining MS/MS were then searched again using the ProLuCID algorithm allowing for the following differential modifications (mass shifts and affected positions/amino acids are indicated in the parentheses): C-terminal amidation (−0.984016, C-terminus), sulfotyrosine (79.956815, Y), pyroglutamic acid (−18.010565, E), gamma carboxyl glutamic acid (43.989829, E), bromotryptophan (77.910511, W), oxidation (15.99491, M), and hydroxylation (15.99491, P, K, V). The raw ProLuCID search results were also filtered and summarized by the DTASelect 2.0 program with a false positive rate of 0% as assessed by reverse hits. For protein quantification, we adopted a generalized G-test using spectral counts from the identified proteins.[29]

RESULTS

LC-MS/MS Analysis

The chromatograms of reduced and alkylated venom samples (Fig. 1) from the three duct regions differ in the number and intensity of peaks indicating a variation of components along the entire venom duct of *C. textile*. Fractionated venom components were introduced into the LTQ-Orbitrap mass spectrometer using electrospray ionization and fragmented using collision induced dissociation (CID). The molecular weights of the peptides were measured by mass spectrometry and tandem mass spectra were recorded at high resolution and with high mass accuracy.[16,18]

Database search using the SEQUEST program identified 24 mature conotoxins that were annotated from *C. textile* (Table 1) and 7 sequences (Table 2) that share close homology to peptides of other *Conus* species. Majority of the full-length conotoxins found in the study belong to the O-superfamily and a few belong to the M and T superfamilies.

Relative Quantification

To quantify the identified conotoxins along the venom duct, a label free approach called spectral counting was employed. Here the number of spectra matched to peptides from a protein is used as a surrogate measure of relative protein abundance.[25] Table 1 and 2 show the total spectral count of all the identified conotoxins in the entire venom duct. The data demonstrate that three members belonging to the O-superfamily of conotoxins predominate among the other venom components. Of these, King-Kong 2 is the most abundant peptide followed by conotoxins TxO4 and TxO6. Our spectral count data are also consistent with the previous studies on *C. textile* venom that showed the conotoxin King-Kong 2 as the most abundant peptide.[30]

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Table 3 highlights relative quantities (spectral count) of representative conotoxins in the various regions of the venom duct. Most conotoxins are highly expressed only in some portions of the venom duct while a few were found in the entire length of the duct. To determine if there is a differential level of expression for each conotoxin in the proximal, central and distal sections of the venom duct, a G-test was employed using spectral count sampling statistics. Computed p-values that are lower than the cutoff ($p < 0.01$) are statistically considered to be differentially expressed along the venom duct. Data analyses (Table 3) show that most *C. textile* conotoxins are differentially expressed in the venom duct. Another interesting feature in the venom is the exclusive expression of certain peptides in only a certain region of the venom duct. Seq. 134 and Tx6.4 are examples of conotoxins that are solely produced in the central region of the duct. This observation suggests that specialization for conotoxin biosynthesis occurs in the different parts of the venom duct. The greatest diversity of conotoxins were found on the central portion (See Figure 2) and 18 of the conotoxins were found throughout the duct.

Identification of Post-Translational Modifications

After database search using SEQUEST, the program ProLuCID was used to search for post-translational modifications (PTM's) considering the known modifications of conotoxins from the Conoserver database. The following amino acid modifications were observed in the conotoxin sequences namely: hydroxylation of proline and valine, bromination of tryptophan, gamma carboxylation of glutamate, C-terminal amidation and pyroglutamation. Though sulfation of tyrosine residues has been documented in *C. pennaceus*[31], none was identified in this study. Methionine oxidation was also observed but was not considered as a modification since this amino acid residue is known to undergo oxidation during the electrospray process in mass spectrometry. Cysteine residues were detected as acetamido derivatives and no alkylated histidine was identified. In Table 4, conotoxins that contain PTM's are compared with respect to total spectral counts of the unmodified peptide and post-translationally modified variants. 7 out of 15 conotoxins have a higher spectral count in their post-translational modified state. Unmodified forms of TxMMSK-02 and TxMEKL-0511 were not observed in this study.

The sequences of the PTM variants are summarized in Table 5. In this study, 14 unreported post-translationally modified peptides (labeled "**This work $**") were found such as the bromination of tryptophan residues in Tx3h, TxMEKL-0511 and Seq.134, γ-carboxylation of glutamate in TxMEKL-022, TxMEKL-0511 and Seq. 134, pyroglutamation of Tx3a variant, and hydroxylation of valine in Tx3a andTxMLKM-021). Hydroxyvaline has been reported in the 8-residue conopeptides called conophans, where HyVal is adjacent to a D-Trp.[32] This is the first time that HyVal is reported in a disulfide-rich conotoxin.

DISCUSSION

Peptide profiling of *Conus* venom has been used as a method to unravel the complexity and examine the intraspecific variation of conopeptides.[37,38] In this study, a proteomics approach was used to identify conotoxins in the *C. textile* venom, to determine their levels of expression in different sections of the venom duct and to determine post-translational modifications. This study takes advantage of high mass resolution and high mass accuracy with the use of a linear ion trap (LTQ)-Orbitrap hybrid mass spectrometer[16] and the computational tools SEQUEST/ProLuCID to identify 31 conotoxin sequences. In addition, 25 PTM variants of 15 conotoxins with masses ranging from 1000-3900 Da (see Tables 1, 2 and 5) were also detected. (Among these were 7 peptides in *C. textile* that matched to sequences described in US Patents for unspecified *Conus* species and one peptide matched to a sequence from *C. litteratus* (LeDr243). It is possible that LeDr243 is another example of peptides like conopressins that perform regulatory functions and occur widely in different

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species of cone snails.[39-42]Although conopressins may perform other functions in the land snail Lymnea[43] and Aplysia[44], their presence in *Conus* venom suggests an important role in the envenomation strategy of *Conus.*

Using a different extraction procedure, bigger polypeptides have been identified. The number of conotoxins reported in this study is less than the total number of *C. textile* peptides in the Conoserver database[45] and they represent only a subset of the venom proteome at a given time and set of conditions. In a study on pooled *Conus virgo* venom, more components were detected using direct infusion of crude venom extract compared to LC-MS analysis.[46] Thus, the chromatographic step performed in this study could contribute to the decreased number of peptides identified compared to what is in the database.

By using spectral count as a measure of relative abundance, most of the identified conotoxins were observed to be differentially expressed in various parts of the tubular duct apparatus of the specimen. This is consistent with the chromatographic profile (Fig.1) obtained for the proximal, central and distal portions of the venom duct. Although other metabolites such as arachidonic acid and low molecular weight amines[47,48] may contribute to the observed differences in chromatograms, the main components of *Conus* venoms are peptides and proteins.

The sensitivity and resolution of LTQ-Orbitrap provide a powerful tool for looking at venom from single specimens and offer the possibility for future analysis of intra-species variations. The data presented here was obtained using only ~10% of the extract from each of the three duct sections. Recently, the use of LTQ-Orbitrap in determining the sequences of peptides from 7% of the venom of a single *C. textile* specimen was reported.[34] Of course, the results obtained from a single snail cannot be used to give a general statement regarding the behavior of other individuals. However, the data when considered in light of findings from different approaches provide insights, and presents insights to plan for future work.

Some conotoxins were found throughout the specimen's venom duct but many were observed only in the proximal-central or central-distal regions, and a couple of peptides belonging to the O-superfamily were restricted to the central region. Our data on the specific location of Tx3c, Tx3a, Tx3.5 are consistent with a previous report on the expression patterns of specific gene superfamilies in particular regions of the venom duct of *C. textile*.[49] The data presented in this paper support previous findings that certain portions of the venom duct specialize in the production of a specific set of venom peptides. This regional specialization of gene expression, processing and secretion of conotoxins in the venom duct was demonstrated for the first time by Garrett and co-authors[49] using a combination of molecular biology and LC-MS techniques. The biosynthesis of certain conotoxins is probably associated with the specific type of epithelial cells found in different sections of the venom duct.[20] The biological observations of Endean and co-workers[1,19] and our own observations indicate greater toxicity of venom from the proximal portion compared to the distal portion. This could only be true if there is a difference of toxin levels in the two regions.

Though it is not fully clear how the variation in conotoxin expression is taking place, it is possible that cone snails may have adopted differential expression of peptides to alter venom content and generate different types of toxin cabals depending on the environmental situation. A relevant example is the ability of captive *C. geographus* to adapt its predatory behavior depending on the nature of prey and hunting environment. The geography cone utilizes different toxin cabals to hunt a single prey or to hunt a school of fish.[50]

In this study on *C. textile*, three major superfamilies were observed in the venom. The O-superfamily to which King Kong 2 belongs has the highest spectral count followed by M-

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superfamily and T-superfamily. Along with the other peptides in Table 2, all of these conotoxins work in concert to affect the various molecular targets of victims. *Conus textile* is a mollusk hunter that preys on other snails in their habitat. The availability of these food resources for *C. textile* may be dictated by oscillating water currents, daily tidal fluctuations and other changes in the overall coral reef system that affect the availability of prey and presence of marine competitors. The capability of *C. textile* to adapt to its niche in this fluctuating environment apparently depends on the evolution of its many toxins that are involved in prey immobilization, defense and competition with marine organisms. Most probably, the venom composition can vary depending on the snail's physiological state and environmental conditions.

It is tempting to speculate that even if an arsenal of peptide-encoding genes exist, only a given set of peptides are expressed or processed for a particular envenomation event. This intricate behavioral repertoire of *Conus*, which is associated with its shifting environment, may be the primary factor that drives a differential expression of venom components.[50] This “selective expression” strategy in *C. textile* could result in a number of neurotoxic peptide cocktails that may successfully allow them to shift depending on prey availability or offer protection from a vast number of changing predators or competitors.

As in other cone snails, the venom apparatus of *C. textile* (Figure 3) includes a venom bulb, a long convoluted venom duct (stretched to show the proximal, central and distal sections), a radula sac that may contain up to 30 harpoons at different stages of formation and a proboscis. In *Conus catus*, as the cone prepares to sting, the radular tooth has been observed to be first positioned in the mid section of the proboscis then propelled by a high-speed ballistic mechanism to hit the prey.[51] The snail's venom bulb is a muscular organ with a thick wall and a lumen filled with clear liquid. Attached to the posterior end of the duct, the bulb is believed to be involved in the transport and delivery of the potent venom components. It may be able to control the volume of venom injected and determine whether the components injected come only from the distal section or includes components from the central and proximal sections. Cone snails generally inject prey a few times as suggested by the presence of several harpoons in the regurgitate of fishbones and scales. The composition of venom injected might vary with successive injections. Delivering an appropriate amount and a proper combination of conotoxins at each injection may be a key determinant in producing the intended physiological response to subdue the prey. It is also noteworthy to take into account the diversity of families and superfamilies of identified conotoxins with each member targeting a specific ion channel or receptor. As stated by Jones et al. (2000)[52] the combinatorial strategy of *Conus* venom presents a perfect scheme for the successful ecological adaptation and envenomation of prey and competitors by cone snails.

Another possible level of control and source of diversity/versatility is the post-translational modification of peptides (PTM) in the *Conus* venom. The spectral counts of both unmodified and posttranslationally modified conotoxins given in Table 4 show the quantitative variation in extent of processing. The varying levels of modification for each conotoxin could represent underprocessed venom component or deliberately produced variants to increase conotoxin diversity. It would be interesting to find out if cone snails have the ability to regulate the degree of posttranslational processing so that a larger conotoxin pool will be accessible to a combinatorial strategy for envenomation. The PTM variants of conotoxins could offer additional diversity to the myriad of constituents present in *Conus* venom and might provide additional leverage for the ecological adaptation of these snails. However, further studies should be conducted on the exact role of the PTM variants since the sample analyzed was obtained on venom squeezed out of duct segments and not from milked venom.

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One interesting set of peptides found in the present study is shown in Figure 3. Here the previously reported Tx3a precursor[33] are shown along with Tx3a[11,34], Peptide 3 (Tx3a analogue with a hydroxylated valine and Gly instead of amide group at the C-terminus) Peptide 4 (Tx3a containing a hydroxylated valine and an amidated C-terminus) and Peptides 1 and 2 which have additional 10 residues at the N-terminus of Tx3a. The arrows on the Tx3a precursor mark the putative cleavage sites for generating the smaller peptides. Are these alternative cleavage sites in the processing of the Tx3a precursor to generate two conotoxins or is Site 1 cleaved as a result of non-specific proteolytic digestion? However, it is interesting to note that Peptide 2 resulting from cleavage at Site 1 has the PTM pyroglutamate at the N-terminus, which tends to stabilize peptides.

The specificity of posttranslational modifications observed in this study has been found in many *Conus* peptides.[53] One PTM variant of the Textile convulsant peptide (Table 5) that was identified in this study contain five proline residues but only the proline at position 12 underwent hydroxylation. This is consistent with a recent study on TxMEKL-P2 where only Trp-19 was brominated although Trp is found also at position 18.[34] What governs this PTM selectivity at present remains unclear at present. A study on Conantokin-G precursor demonstrated that the propeptide region has a potential function of being a recognition sequence for the *Conus* venom enzyme γ-glutamyl carboxylase.[55] Recognition signals in the pro-region may specify the amino acid residues to be modified in a conopeptide sequence.[53]

Proteomics provide insights on the biochemical events involved in the modification process. A typical example of such is the C-terminal amidation of several conotoxins of *C. textile*. ProLuCID identified C-terminal amidation of several conotoxins (see Table 5) while SEQUEST detected their C-terminal acid counterparts (see Table I). Our data are consistent with the enzymatic conversion of glycine to a C-terminal amide group. The data on the two PTM variants of TxIIIC containing their pro-regions confirm that modification such as hydroxylation and C-terminal amidation can take place before cleavage of the precursor region of the conotoxin. Previous studies have reported TxIIIC to contain two PTM's.[11,34] The current work validates the previous findings and also identified the modifications in both the precursor and mature peptide.

Posttranslational modification is known to perform several roles in conopeptides. In some cases such as γ-carboxylation in conantokin-G, modification is extremely important for activity[56], in other cases posttranslational modification could improve the functional efficiency of the conopeptides.[53,54,57,58] Studies on tx9a and gm8a peptides[57] showed that an alternative role to be considered is the facilitation of correct folding of conotoxins particularly when modification occurs early in the secretory process.[53] Perhaps another possible role is to stabilize the toxin from enzymatic degradation, as in the racemization of L- to D-tryptophan in contryphans[60] and cylization of N-terminal glutamate to pyroglutamate[57,61,62] in *C. geographus* and *C.striatus*. According to a recent review by Bingham and co-authors[63], PTMs have evolved to provide maximal targeting function from a single translational gene product and greater response to evolutionary changes and environmental demands.

The study has provided a number of insights regarding venom processing, However, the results raised a number of questions that could be answered only by comparing the composition of crude venom from the duct with milked venom. The venom actually injected by cone snails has been reported to contain fewer components than that extracted from the duct and ~50% of the component masses found in milked venom of *C. consors* were not detected in the venom obtained by dissection[64]. It has been suggested that some of the component masses found only in the milked venom could be produced by the salivary gland[65] and by the epithelial zones between the venom duct and pharynx[20]. However, it is

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certainly possible that a significant amount of the component masses distinct to the milked venom could be PTM variants that are produced as the venom is further processed from the time the cone snail detects and hunts its prey until the venom passes from the duct to the proboscis and harpoon. This might involve enzymes produced in the venom duct as well as in the salivary gland and epithelial regions of the pharynx.

CONCLUSION

This study takes advantage of developments in mass spectrometry-based proteomics to gain insights on the envenomation strategy used by *C. textile* in its predatory behavior. Analysis of the venom using the LTQ-Orbitrap and database searches revealed qualitative and quantitative differences in conotoxin components in the various sections of the venom duct of *C. textile*. Our findings are consistent with previous reports on the diversity of conotoxins. The data in this study suggest that PTM variants can greatly enrich the peptide pool for the combinatorial strategy used in envenomation. The specialization of conotoxin biosynthesis in different sections of the venom duct, the control of the level of post-translational modification may contribute to the production of toxin cabals appropriate to a particular predatory or defense situation.

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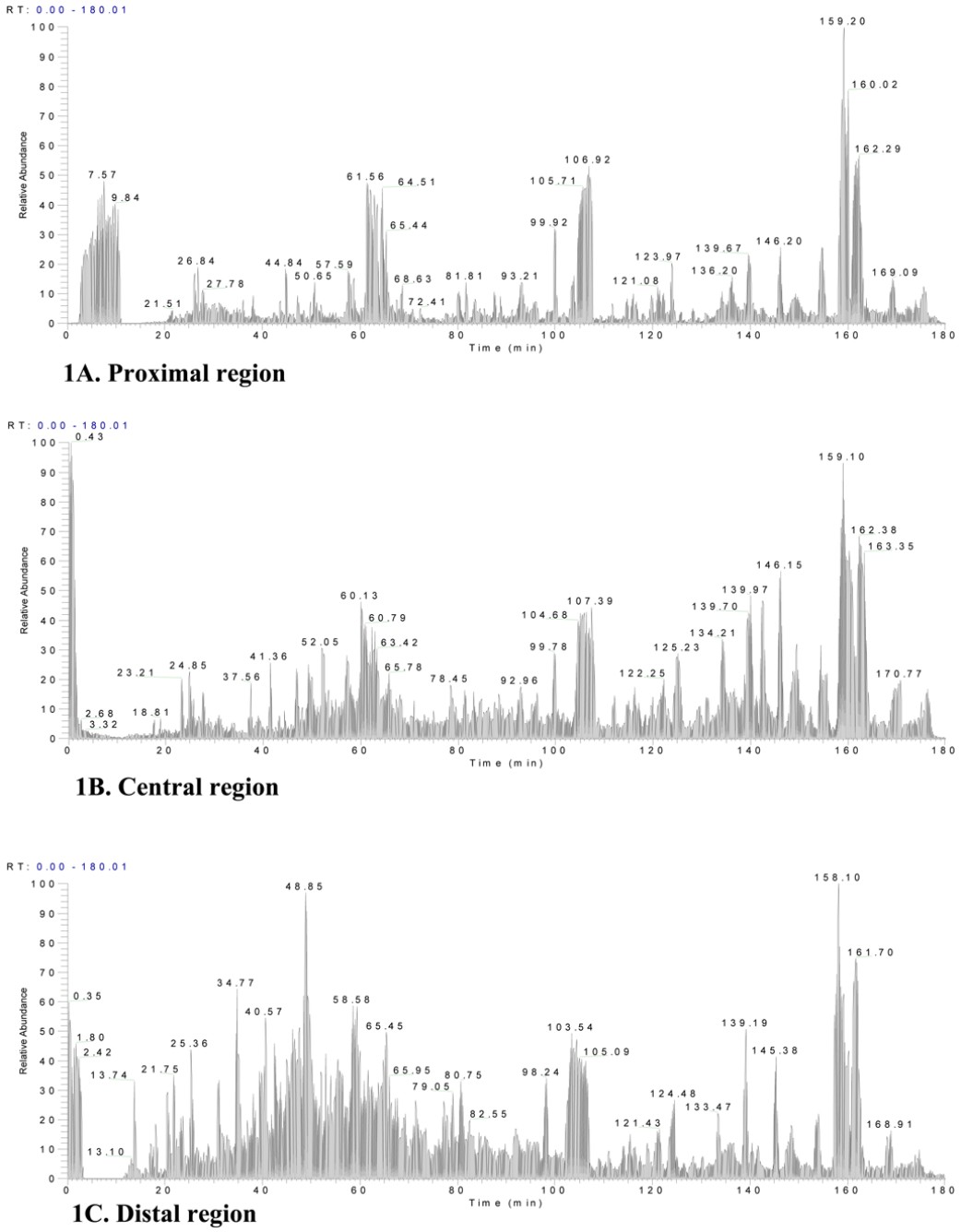

Figure 1.
Chromatograms of *Conus textile* venom from three sections of the duct

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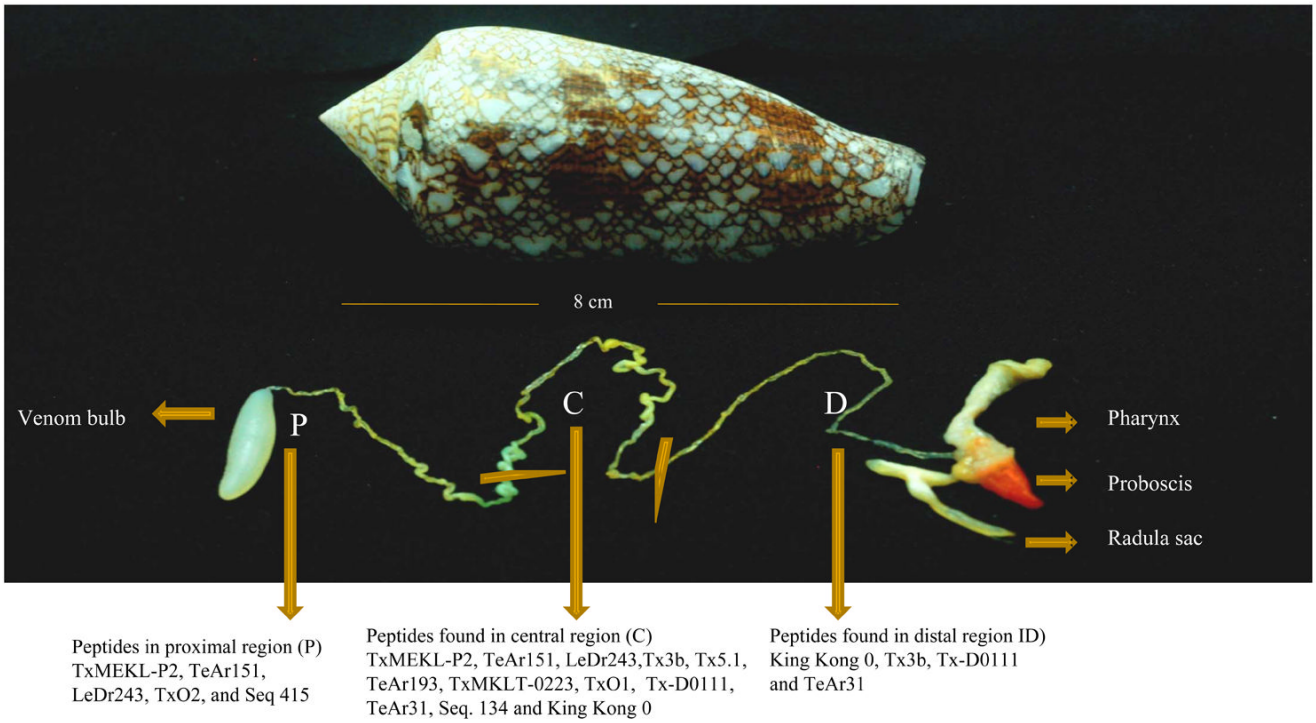

Tx*- peptides found in all regions of the venom duct : Textile convulsant peptide, TxO6, TxO4, TxMKLT-015, Tx3.5, TxMRCL-02, King Kong 2, Tx3.4, Tx3h, TxMLKM-021, TxMEKL-022, Tx3a peptides, Seq 222, Seq. 16 USPN 6762165, Seq 67, TxMLKT1-031 and Seq . 67 USPN 6767896

Figure 2.

Distribution of conotoxins in the venom duct.. The radula sac contains about 30 hollow chitinous harpoons at different stages of formation. (Photo courtesy of Prof. Gil S. Jacinto and Katrina S. Luzon of UP Marine Science Institute.)

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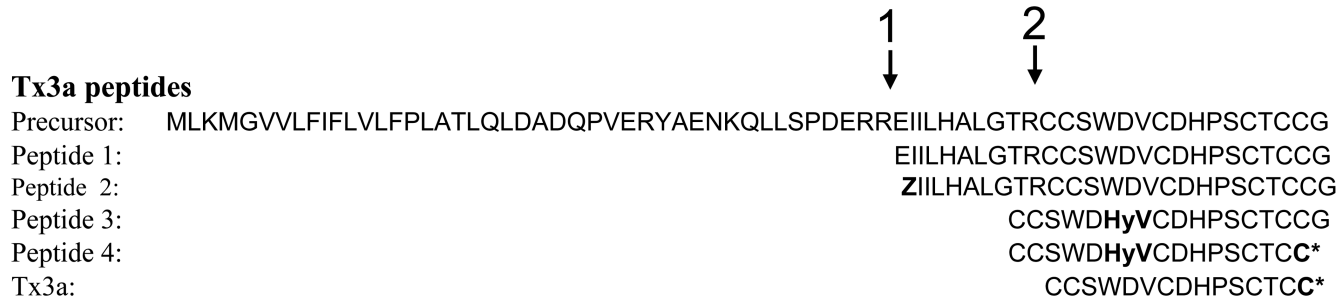

Figure 3.

Alternative cleavage sites on the propeptide of Tx3a and three resulting conotoxins. Note: **Z** represents PyroGlu, **HyV** is a hydroxylated valine and **C*** is an amidated Cys residue.

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Table 1

Summary of mature conotoxins in Conus textile venom identified by mass spectrometry from annotated C. textile database.

Conotoxin	Sequence	Spectral Counts
O-superfamily		
King-Kong 2	CAPFLHPCTFFFPNCCNSYCVQFICL	162
TxO4/TxMKLT-0221	YDCEPPGNFCGMIKIGPPCCSGWCFFACA	125
TxO6	NYCQEKWDYCPVPFLGSRYCCDGLFCTLFFCA	116
TxMKLT1-015	CIEQFDPCDMIRHTCCVGVCFLMACI	35
TxIA/TxVIA KingKong 0	WCKQSGEMCNLLDQNCCDGYCIVLVCT	22
Textile convulsant peptide	NCPYCVVYCCPPAYCEASGCRPPG	19
TxMEKL-022	WREGSCTSWLATCTDASQCCTGVCYKRAYCALWE	19
TxMKLT-0223/Tx6.4	DDCEPPGNFCGMIKIGPPCCSGWCFFACA	17
TxMEKL-P2	RGYDAPCSSGAPCCDWWTCSAR	12
TxMKLT1-031	DCQEKWDFCPAPFFGSRYCCFGLFCTLFFCA	7
TxO2	CYDSGTSCNTGNQCCSGWCIFVCL	5
TxO1/TeA61	RCLDAGEVCDIFFPTCCGYCILLFCA	2
	Total	541
M-superfamily		
Tx3a/Tx3a variant	CCSWDVCDHPSCTCCG / EIILHALGTRCCSWDVCDHPSCTCCG	94
Tx3h	KFCCDSNWCHISDCECCYG	88
Tx3.5	RCCKFPCPDSCRYLCCG	58
TxMLKM-021	VCCPFGGCHELCQCCEG	25
Tx3.4/TxIIIC/Tx3c/scratcher peptide	CCRTCFGCTPCCG	10
Tx3b/Tx3.3	CCPPVACNMGCKPCCG	9
	Total	284
T-superfamily		
TxMRCL-02	TSDCCFYHNCCC	32
TeAr151	VCCRPMQDCCSG	17
TeA31	KRICCYPNVWCCD	8
Tx-D0111	QCCWYFDISCCITV	7
Tx5.1	RCCQTFYWCCVQG	4
TeAr193	NCCRRQICCG	4
	Total	72

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Table 2

Summary of mature conotoxins in Conus textile venom identified by mass spectrometry homologous to those of other Conus species.

Conotoxin	Superfamily	Sequence Identified by Mass Spectrometry	Spectral Counts
Seq.222 USPN 6762165	O	…CQEKWDYCPVPFLGSRYCCDGFICPSFFCA…	86
Seq. 16 USPN 6762165	O	…KEHQLCDLIFQNCCRG…	63
Seq. 134 USPN 6762165	O	…NTRDYDCEPPGNFCGMIKIGPPCCSGWCFFACA…	41
Seq. 67 USPN 6797808	A	…KGCCSRPPCIANNPDLCG…	34
LeDr243	T	…RKPCCSIHDNSCCG…	27
Seq. 16 USPN 6767896	A	…KQTCCGYRMCVPCG…	13
Seq. 415 USPN 6767895	I	…RGTCSGRGQECKHDSDCCGHLCCAGITCQFTYIPCK…	8

Note:

a) USPN: United States Patent Number

b) … Indicates possible sequence extensions.

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Table 3

Summary of representative conotoxins and their relative quantities in the regions of the venom duct.

	Spectral Counts		
	Proximal	**Central**	**Distal**
Conotoxins more abundant in the proximal region			
LeDr243 *	20	7	0
Textile convulsant peptide *	10	3	6
Seq. 222 USPN 6762165 *	49	18	19
King-Kong 2*	82	42	38
TxO4/TxMKLT1-0221 *	63	37	25
TxO6 *	49	29	38
Conotoxins more abundant in the central region			
Seq. 134 USPN 6762165 *	0	41	0
TxMKLT-0223/Tx6.4 *	0	17	0
TeAr151 *	3	14	0
TxIA/TxVIA KingKong 0 *	0	14	8
TxMLKM-021*	2	16	7
TxMEKL-P2*	5	7	0
Conotoxins more abundant in the distal region			
Seq. 16 USPN 6762165 *	2	4	57
Seq. 67 USPN 6797808 *	12	10	22
Tx3.1 *	26	28	34
Conotoxins more abundant in two regions of the venom duct			
Tx3a peptides*	44	38	12
TxMRCL-02 *	13	13	6
Tx3.5 *	24	8	26
TxMKLT1-015 *	6	14	15
Conotoxins more or less evenly distributed in all regions of the venom duct			
TxMEKL022	6	8	5
Tx3.4/TxIIIC/Tx3c/scratcher peptide	2	5	3

Note:

* Indicates differentially expressed peptides (p-value < 0.01) using G-test.

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Table 4

Comparison of Spectral Counts of Unmodified and Post-translationally Modified Conotoxins

Conotoxin	Spectral Count		No. of Different PTM Variants
	Unmodified Peptides	Peptides with PTM's	
Peptides with higher spectral count in the unmodified states			
Textile Convulsant peptide	19	4	2
TxMLKM-021	25	10	2
TxMEKL-022	19	3	1
Tx3a/Tx3.2 peptides	94	23	4
Tx3h	88	29	3
Tx3b/Tx3.3	9	4	1
Tx3.5	58	8	1
Tx5.1	4	2	1
Peptides with higher spectral count in the post-translationally modified states			
TxIIIC/Tx3C	10	20	3
TxMEKL-P2	12	24	1
TeA31	8	26	1
Seq.134 USPN 6762165	41	90	2
TeAr151	17	21	1
TxMMSK-02	0	6	1
TxMEKL-0511	0	17	1

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Table 5

Summary of Identified Post-translational Modifications and their Sequences

Conotoxin	Sequence	References
TxMEKL-022	WREGSCTSWLATCTDASQCCTGVCYKRAYCALWE	33
	WRγGSCTSWLATCTDASQCCTGVCYKRAYCALWE	**This work** $
TxMEKPL-2	DCRGYDAPCSSGAPCCDW**W**TCSARTNRCF	34; This work
Tx3b/Tx3.3	CCPPVACNMGCKPC**C***	11; This work
Tx3.5	RCCKFPCPDSCRYLC**C***	11; 34; This work
TeAr151	VCCRPMQDCC**S***	34; 35; This work
Tx5.1	RCCQTFYWCCV**Q***	14; This work
TeA31	RICCYPNV**W**CCD	34; 35; This work
Textile Convulsant peptide	NCPYCVVYCCPPAYCEASGCRP**P***	7; 10; This work
	NCPYCVVYCCP**O**AYCEASGCRP**P***	**This work** $
TxIIIC	CCRTCFGCT**O**C**C***	11; 34; This work
	LLDDISFENNPFYDPAKRCCRTCFGCTPC**C***	**This work** $
	LLDDISFENNPFYDPAKRCCRTCFGCT**O**C**C***	**This work** $
TxMLKM-021	VCCPFGGCHELCQCC**E***	34; This work
	HyVCCPFGGCHELCQCC**E***	**This work** $
Tx3a/Tx3.2 peptides	CCSWDVCDHPSCTC**C***	11; 33; 34; This work
	CCSWD**HyV**CDHPSCTCCG	**This work** $
	CCSWD**HyV**CDHPSCTC**C***	**This work** $
	ZIILHALGTRCCSWDVCDHPSCTCCG	**This work** $
TxMMSK-02	CCDDSECSYSCWPCCYG	33
	CCDDSECSYSCW**O**CCYG	**This work** $
TxMEKL-0511	WWRWGGCMAWFGLCSKDSECCSNSCDVTRCELMPFPPDW	33
	WWR**W**GGCMA**W**FGLCSKDSγCCSNSCDVTRCELMPFPPDW	**This work** $
TxMLKM-011/Tx3h	KFCCDSNWCHISDCECC**Y***	11; 34
	KFCCDSN**W**CHISDCECCYG	**This work** $
	KFCCDSN**W**CHISDCECC**Y***	**This work** $
Seq. 134 USPN 6762165	MKLTCLMIVAVLFLTAWTFVTAVPHSSNALENLYLKARHEM	36

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Conotoxin	Sequence	References
	ENPEASKLNTRDYDCEPPGNFCGMIKIGPPCCSGWCFFACA	
	DYDCEPPGNFCGMIKIGPPCCSG**W**CFFACA	**This work** $
	DYDCγPPGNFCGMIKIGPPCCSG**W**CFFACA	**This work** $

Note: The following symbols are used to designate posttranslational modifications

γ- Gamma carboxylation

Z-pyroglutamation

O-hydroxyproline

* C-terminal amidation

HyV- hydroxyvaline

W - bromotryptophan

$ Indicate unreported PTM variants