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Biochemical and gene expression analyses of conotoxins in *Conus textile* venom ducts

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Abstract

Each *Conus* snail species produces 50–200 unique peptide-based conotoxins, derived from a number of different gene superfamilies. Conotoxins are synthesized and secreted in a long venom duct, but biochemical and molecular aspects of their biosynthesis remain poorly understood. Here, we analyzed expression patterns of conotoxin genes belonging to different superfamilies in *Conus textile* venom ducts. The results demonstrate that specific gene families are expressed in particular regions of the venom duct. Biochemical analysis using liquid chromatography and mass spectrometry revealed an even more localized accumulation of individual conotoxins. This study demonstrates for the first time that specialization of gene expression, processing, and secretion of conotoxins occurs in different regions of the venom duct.

Keywords: Conotoxins; *Conus*; Cone snails; Biosynthesis; Conotoxin gene expression; Venom duct

Conotoxins represent a unique group of peptide-based neurotoxins produced by predatory marine snails from the genus *Conus* [1]. It is generally accepted that each of estimated 500–700 different *Conus* snails produces 50–200 distinct conotoxins, yielding an impressive repertoire of ~100,000 unique peptides. Most of these peptides are crosslinked by disulfide bonds that stabilize the biologically active conformation. Based on gene homology and conserved toxin structure, conotoxins can be divided into distinct superfamilies and families. Conotoxins are biosynthesized as larger precursor polypeptides, consisting of the N-terminal signal sequence, intervening propeptide, and a mature toxin [2]. It is conceivable that cone snails have evolved a set of specialized biochemical and cell biological adaptations for processing these unusual gene products throughout the secretory pathway [3–5]. Compared to more conventional gene products, conotoxins are unusually small but highly structured. A further characteristic feature of conotoxins is a remarkable number of posttranslational modifications, such as O-glycosylation, bromination of tryptophan, γ-carboxylation of glutamate residues, hydroxylation of prolines or L- to D-epimerization [6].

The unprecedented molecular diversity of these gene-based natural products posed several intriguing questions related to their biosynthesis. In particular, biochemical and cellular aspects of generating multiple posttranslational modifications in the specialized *Conus* snail venom apparatus remain poorly understood [7–9]. Little is known about the oxidative folding of individual conotoxins in the endoplasmic reticulum, enzymes

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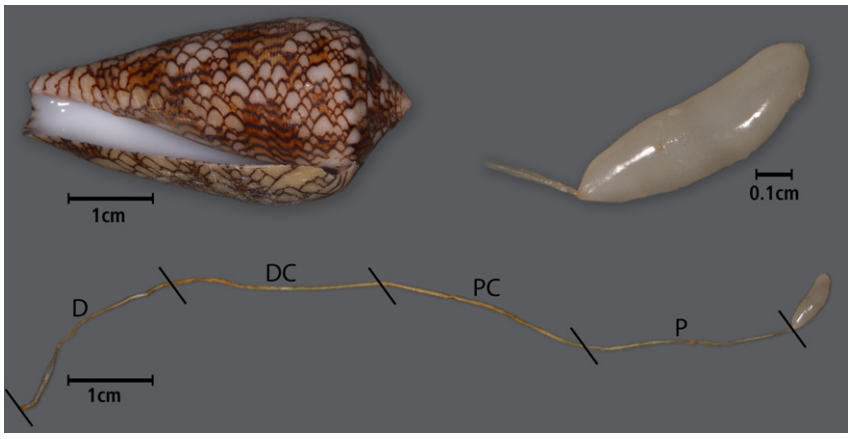

Fig. 1. *Conus textile* shell and dissected venom duct. The venom duct was dissected and split into four equal segments, labeled D (distal), DC (distal/central), PC (proximal/central), and P (proximal). The proximal segment is connected to the muscle bulb (enlarged). The distal segment is connected to the pharynx of the snail. The venom is injected through a harpoon-like hollow tooth.

involved in introducing posttranslational modifications in Golgi apparatus, and about trafficking of conotoxins in the secretory pathway [10–13]. The subcellular localization of proteolytic processing of the conotoxin precursors remains unknown. At the end of the secretory pathway, conotoxins are packed into the characteristic, football-shaped secretory granules that are secreted into the duct lumen [14]. All of these biosynthetic steps occur in the long, convoluted venom duct (Fig. 1).

At present, the diversity of conotoxin-producing secretory cells within a venom duct is unknown. A recent study by Marshall et al. [9] suggested that the proximal part of the venom duct might be specialized in active transport rather than secretion of conotoxins. In this work, we investigated expression of conotoxin genes in discrete segments of a venom duct from *Conus textile*. Our results show that biosynthesis and secretion of individual conotoxins belonging to different superfamilies occur in discrete parts of the venom duct, suggesting functional specialization. A definition of the degree of specialization in the secretory cells producing conotoxins is prerequisite to obtaining insight into how uniquely, cone snails have evolved such a remarkable diversity of what are among the smallest functional gene products known.

Materials and methods

Construction of cDNA libraries and RT-PCR. Venom ducts from individual adult *C. textile* specimens were dissected on ice, divided into four equal lengthwise segments, and frozen at −80 °C until used for RNA preparation. Total cellular RNA was isolated from each individual venom duct tissue segment sample using the Trizol procedure and RNA yield was quantified by spectrometry. Equivalent amounts of total cellular RNA (15 μg) from each venom duct segment sample were used in the synthesis of first-strand cDNA using an oligo(dT) primer and Superscipt II reverse transcriptase (Invitrogen) according to the supplier's protocol.

For RT-PCR analysis of conotoxin gene expression, primers were designed based on sequences found in the GenBank database for each of the conopeptide families. For each conopeptide family, the primer pair was designed to amplify the complete open reading frame of the conotoxin precursor protein, and would generally amplify a product of ~250–350 bp. To amplify the *Conus* protein disulfide isomerase and actin products, primers were designed to amplify short regions within the coding regions of the respective mRNAs. For all of the PCR primer pairs, we have previously used these primers in the cloning and sequencing of conotoxin gene products, thereby confirming the specificity of the products that they generate. PCR was performed using 200 ng cDNA in each reaction, using *Taq* polymerase with buffer components provided by the supplier (Promega). PCR cycling was performed with 30 total cycles, and reaction products were analyzed by electrophoresis on 2% agarose gels.

Identification of conotoxin sequences. Cloning and sequencing of conotoxin genes was performed as previously described [15,16].

Venom peptide extraction and HPLC analysis. The venom ducts from live *C. textile* were dissected on ice and immediately divided into four equal segments (Fig. 1). Each part of the venom duct segment was ground under liquid nitrogen. Extraction was performed in 1 ml of 20% acetonitrile, 0.1% TFA at 4 °C. After mixing for 1 h, the extract solution was centrifuged. The resultant pellet was resuspended in 1 ml of 10% acetonitrile, 0.1% TFA and again mixed for 1 h at 4 °C, followed by centrifugation. The supernatants from the first and second extraction were pooled. The 20 μl of extracted venom from each part of a duct was loaded on a Vydac C_{18} analytical HPLC column, and the peptides were separated at the column temperature of 45 °C. Samples were eluted using solvents A (0.1% TFA) and B (90% acetonitrile and 0.1% TFA), and mixed to form a linear gradient of 10–100% solvent B, for 45 min. The collected fractions were analyzed by matrix-assisted laser desorption–ionization (MALDI) mass spectrometry. The 10 μl of extracted *C. textile* venom was loaded on a Vydac C_{18} HPLC column. Samples were eluted using solvents A (0.01% TFA) and B (90% acetonitrile and 0.01% TFA), and mixed to form a linear gradient of 10–100% solvent B, for 1 h, with 0.2 ml/min flow rate. Eluting components were directly infused into Micromass Quattro II—triple quadrupole mass spectrometer. Both total ion intensity and molecular ion species were recorded simultaneously.

MALDI mass spectrometry. Matrix-assisted laser desorption ionization (MALDI) mass spectra were obtained using a Brucer REFLEX time-of-flight mass spectrometer (Bruker Daltonics) fitted with gridless reflectron, an N_2 laser, and a 100 MHz digitizer, courtesy of the Salk Institute for Biological Studies (La Jolla, CA, USA).

Peptide sequencing. The standard automated Edman degradation was performed on Applied Biosystems Model 492 Sequenator, courtesy of Dr. Robert Schackmann of the DNA/Peptide facility, University of Utah.

Results and discussion

To explore how conotoxins are expressed in *Conus* snail venom ducts, we employed parallel analyses using: (1) RT-PCR of mRNA coding different superfamilies of conotoxins, and (2) HPLC separation, mass spectrometry, and N-terminal sequence analysis of extracted conotoxins to determine the distribution of conotoxin families and individual peptides in four distinct parts of a venom duct from *C. textile*. Fig. 1 illustrates a dissected venom duct of *C. textile* and its separation into four segments: (1) distal, D; (2) distal/central, DC; (3) proximal/central, PC; and (4) proximal, P. It is generally accepted that the muscular bulb at the posterior end of the venom duct does not play a role in secretion of conotoxins [7,9] (J.E. Garrett, unpublished results).

RT-PCR analysis revealed regional variation in the expression of specific conotoxin gene families along the length of the venom duct (Fig. 2). Amplification of the actin gene served as a control to demonstrate equivalent levels of cDNA input from each of the duct segment RNA preparations. All conopeptide families examined were abundantly expressed in the proximal duct segment, with expression levels decreasing for some of the gene families in the distal/central and distal segments. Only the O-superfamily displayed robust expression in the distal segment, and in fact, stronger expression levels of a larger O-family gene product were evident in this segment.

We previously showed that a specialized folding catalyst, protein disulfide isomerase (PDI), was found as a very abundant polypeptide in the *C. textile* venom duct [10]. Here, we examined expression of PDI genes in the corresponding segments of the venom duct. As shown in Fig. 2, RT-PCR using PDI-specific primers yielded equivalent levels of cDNA input from each of the duct segment RNA preparations. These observations are in accord with our previous biochemical analysis of PDI from discrete segments of the venom duct, indicating that this enzyme is expressed throughout the whole length of the duct [10].

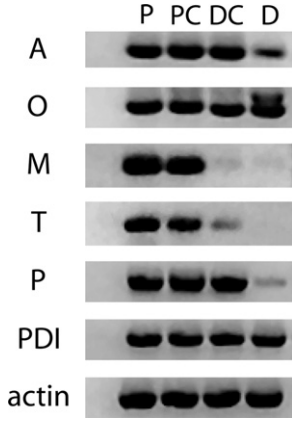

Fig. 2. RT-PCR analysis of expression of mRNA for conotoxins from different superfamilies. A, O, M, T, and P correspond to the A-, O-, M-, T-, and P-superfamily of conotoxins. Protein disulfide isomerase and actin (control) were also included in the analysis.

It should be noted that for each conotoxin gene family analyzed in Fig. 2, several different genes may be concurrently expressed in *C. textile*. The family-specific PCR primers will amplify all of these family members, and the apparent expression of a gene family across several duct segments may represent expression of multiple individual genes within a family. Expression of an individual family member may be more tightly restricted than the overall family as a whole, as appears to be the case with the larger O-family product, which is strongly expressed only in the distal segment. A number of individual conotoxins belonging to these superfamilies have previously been identified: T-superfamily [17,18] (B.M. Olivera and M. Watkins, unpublished results), O-superfamily [19,20], P-superfamily [21], and M-superfamily [22,24].

To determine the distribution of individual conotoxins throughout the venom duct, we carried out an extraction of conotoxins from each segment. The extracts were analyzed using reversed-phase HPLC separations using two different types of detection: UV absorbance at 220 nm or electrospray-ionization mass spectrometry (ESI-MS). Major peaks from the UV-monitored HPLC separations were collected and further analyzed by MALDI-TOF and N-terminal sequencing. Fig. 3 summarizes HPLC analysis of venom extracts from the four parts of the duct, D, DC, PC, and P. HPLC peaks corresponding to conotoxins were observed in all four extracts; however, the distribution of conotoxins differed significantly. Both PC and DC extracts were characterized by a major peak with a molecular mass of 3035, corresponding to that of previously discovered δ-conotoxin TxVIA. Indeed, we confirmed the identity of this peptide by coelution with the reference standard (synthetic δ-TxVIA prepared as described in [23]).

Additional conotoxins were identified by microsequencing of the N-terminus and matching the molecular mass (determined by MALDI-TOF) with those predicted from cDNA sequencing. Identification of additional conotoxins, based on their molecular masses, was confirmed by LC/MS experiments (Fig. 4). These results are summarized in Table 1. Three additional δ-conotoxins, namely Tx6.3, Tx6.8, and Tx6.14, were identified. The sequences of δ-conotoxins were determined from the cDNA libraries prepared as previously described [16]. Calculated molecular masses of the predicted mature toxins agreed with those obtained by

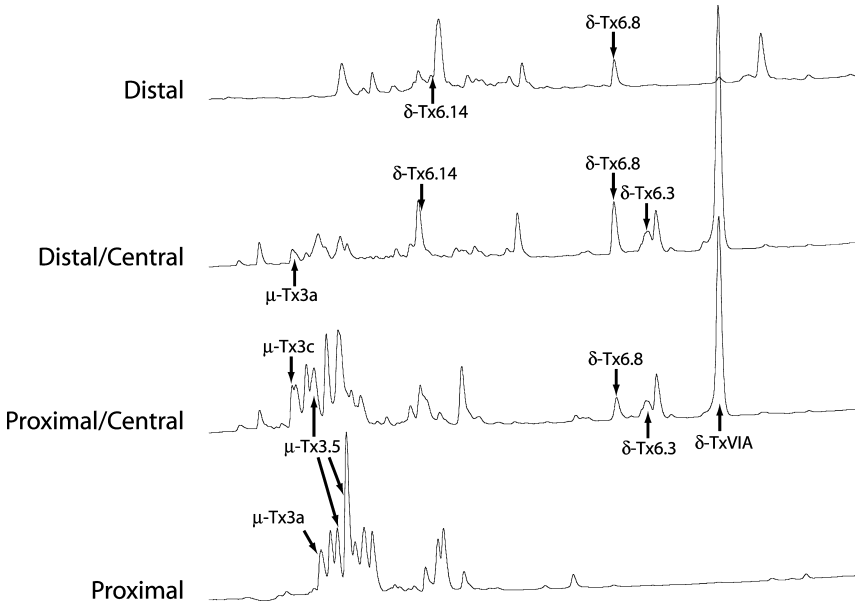

Fig. 3. HPLC analysis of conotoxins extracted from four segments of the venom ducts. The extracted conotoxins were separated using reversed-phase C_{18} column. The elution was monitored by measuring the UV absorbance at 220 nm. Labeled HPLC peaks correspond to conotoxins identified by mass spectrometry or/and microsequencing.

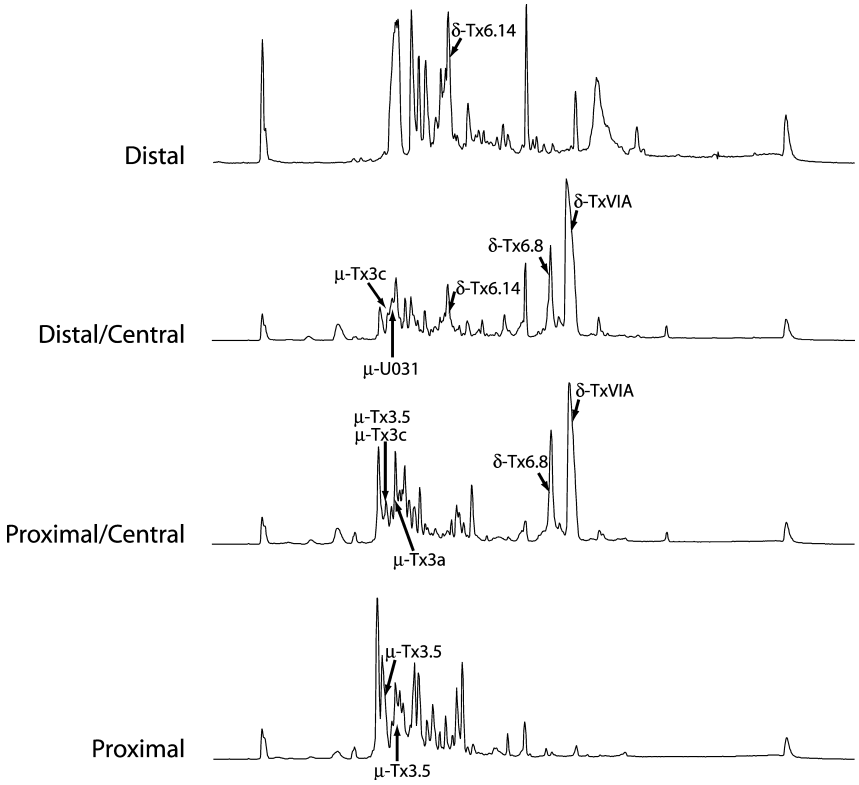

Fig. 4. LC/MS analysis of conotoxins extracted from four segments of the venom ducts. The same samples were analyzed as shown in Fig. 3. The conotoxins were separated using identical reversed-phase conditions, except 0.01% TFA was used as a counter ion. Elution was monitored using electrospray-ionization mass spectrometry by a continuous scanning in the mass range from 200 to 2000.

MALDI-TOF or with those obtained from the LC/MS analysis (Fig. 4). Like δ-TxVIA, all three δ-conotoxins were the most abundant in the central segment of the venom duct. All δ-conotoxins belong to the O-superfamily; since approximately equal expression of O-superfamily mRNA was found in all four segments

Table 1
Identification of individual conotoxins in the venom duct of *Conus textile* using mass spectrometry and N-terminal sequencing

Conotoxin	Sequence	MH^+_{calc} (Da)	MH^+_{obs} (Da)
μ-Tx3c	CCRTCFGCTOCC#	1305.38	DC 1305.2 PC 1305.4
μ-Tx3a	CCSWDVCDHPSCTCC#	1654.47	DC 1654.2 PC 1654.5; 1654.6 P 1654.3
μ-Tx3.5	RCCKFPCPDSCRYLCC#	1889.72	PC 1890.7 P 1889.4; 1890.6
δ-TxVIA	WCKQSGEMCNLLDQNCCDGYCIVLVCT^	3035.00	DC 3037.0 PC 3036.97
δ-Tx6.3	KCVEQWKYCTRESLCCAGLCLFSFCIL^	3140.40	DC 3141.3 PC 3142.3
δ-Tx6.8	CYDSGTSCNTGNQCCSGWCIFVCL^	2557.90	D 2558.95 DC 2558.9 PC 2558.8
δ-Tx6.14	DCYSWLGSCIAPSQCCSEVCDYYCRLWR^	3305.78	D 3304.8 DC 3304.7

D, DC, PC, and P: distal, distal/central, proximal/central, and proximal, respectively. # denotes C-terminal amidation, ^ denotes free carboxyl group at the C-terminus.

(Fig. 2), other members of the superfamily must have a complementary distribution.

Three conotoxins from the M-superfamily were identified by MALDI-TOF or LC/MS: Tx3a, Tx3c, and Tx3.5 (Table 1, Figs. 3 and 4). Interestingly, these peptides were detected in the proximal and proximal/central part, consistent with the RT-PCR results. This finding strongly suggests that the proximal part of the venom duct is also actively involved in the expression and secretion of conotoxins, contrary to what was previously suggested [9].

Taken together, our results indicate that conotoxins are expressed and secreted in discrete segments of the venom duct. It is tempting to hypothesize that clusters of secretory cells localized in the different segments are specialized for the expression, processing, and secretion of a small set of conotoxins from a particular family/superfamily. On a practical note, cloning of conotoxin genes from particular superfamilies (and corresponding enzymes involved in their posttranslational processing) can be significantly improved if mRNA is prepared from the appropriate segment of the venom duct, rather than from the whole-length duct. In addition, proteomic-like analysis of conotoxins extracted from individual segments using LC/MS methods may also yield better detection and identification of individual members of conotoxin families. Many conotoxins are posttranslationally modified (reviewed in [6]). It is conceivable that the specialized enzymes responsible for these unusual modifications are also co-expressed in the same group of cells that specialize in biosynthesis of particular conotoxins.

Our studies provide strong evidence for a biochemical and cell-biological specialization within *Conus* venom ducts for the production of these unusually small, rigidly structured, and highly modified gene products. Secretory cells producing conotoxins that belong to different families (that diverge in size, disulfide connectivity, and posttranslational modification) might be expected to have a different set of biochemical specializations. For example, the ability to form a specific pattern of disulfides might require different factors for divergent conotoxin families. The biochemical characterization we have described here is the initial step towards addressing these questions.

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