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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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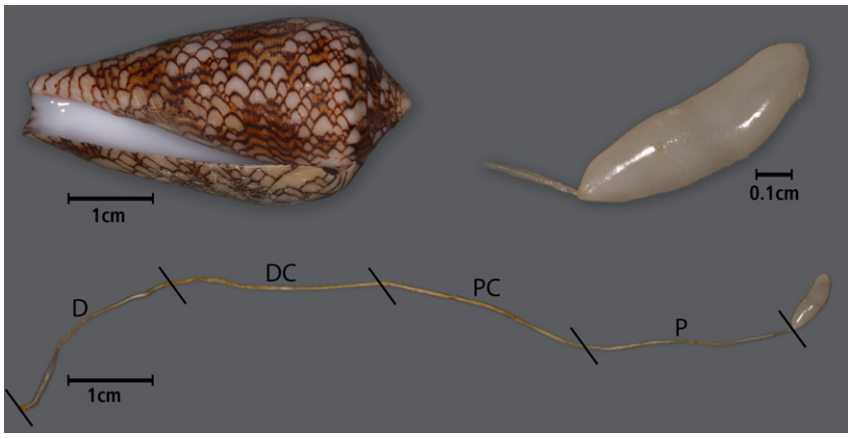

Fig. 1. *Conus textile* shell and dissected venom duct. The venom duct was dissected and split into four equal segments, labeled D (distal), DC (distal/central), PC (proximal/central), and P (proximal). The proximal segment is connected to the muscle bulb (enlarged). The distal segment is connected to 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).