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α-Conopeptides specifically expressed in the salivary gland of *Conus pulicarius*☆

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ABSTRACT

To date, studies conducted on cone snail venoms have attributed the origins of this complex mixture of neuroactive peptides entirely to gene expression by the secretory cells lining the lumen of the venom duct. However, specialized tissues such as the salivary glands also secrete their contents into the anterior gut and could potentially contribute some venom components injected into target animals; evidence supporting this possibility is reported here. Sequence analysis of a cDNA library created from a salivary gland of *Conus pulicarius* revealed the expression of two transcripts whose predicted gene products, after post-translational processing, strikingly resemble mature conopeptides belonging to the α-conotoxin family. These two transcripts, like α-conotoxin transcripts, putatively encode mature peptides containing the conserved A-superfamily cysteine pattern (CC–C–C) but the highly conserved A-superfamily signal sequences were not present. Analysis of A-superfamily members expressed in the venom duct of the same *C. pulicarius* specimens revealed three putative α-conotoxin sequences; the salivary gland transcripts were not found in the venom duct cDNA library, suggesting that these α-conotoxins are salivary gland specific. Therefore, expression of conotoxin-like gene products by the salivary gland could potentially add to the complexity of *Conus* venoms.

1. Introduction

Venoms of predatory gastropods in the genus *Conus* ("cone snails") have been intensively investigated; these venom components have the potential to be useful pharmacological agents that act predominantly on molecular targets in nervous systems (Wang and Chi, 2004; Norton and Olivera, 2006; Olivera, 2006; Olivera and Teichert, 2007). Decades of research have documented that these venoms comprise a complex mixture of secreted gene products synthesized by a specialized organ referred to as the venom duct (Olivera and Cruz, 2001; Olivera, 2002). During envenomation, contraction of the venom bulb causes venom duct's contents to be forced through the proboscis, which is preloaded with a single harpoon-like tooth; the harpoon, which is hollow, is used to inject venom into the target animal (Schulz et al., 2004).

The venom toxins are mostly small, disulfide-rich peptides, whose transcripts encode precursors with a characteristic organization – at the N-terminus is a signal sequence, followed by an intervening "pro" region, which includes a protease cleavage site, and at the C-terminus, the mature toxin, always in single copy and generally rich in cysteine codons (Woodward et al., 1990; Olivera et al., 1999). Within the mature toxin region, only a few characteristic arrangements of cys residues occur; these are

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referred to as "Cys patterns". Each Cys pattern generally specifies a particular disulfide framework, the dominant structural determinants that stabilize the three-dimensional conformation of these unusually small (12–30 AA) peptide toxins. A most remarkable feature of *Conus* toxin precursors from the venom duct is that toxins with a particular Cys pattern also share a highly conserved signal sequence. The combined presence of these superfamily specific characters distinguishes particular conopeptide gene superfamilies (Terlau and Olivera, 2004).

In *Conus* and other Conoidean gastropods, other secretory organs, such as the salivary or accessory salivary gland, can potentially secrete gene products into the lumen of the anterior gut (Taylor et al., 1993). Therefore, these glands may also contribute their own gene products to the venom. Although no previous studies have addressed what gene products are produced by the salivary glands of *Conus*, the salivary secretions of a closely related family of carnivorous snails (i.e., the Turrids) have been postulated to perform one or more functions, which include: (i) increasing envenomation efficiency during prey capture, either by proteolytically removing debris, or lubricating the radular tooth, while preparing for hypodermic injection; (ii) proteolytic processing of venom components just prior to, or at the site of injection via events resembling the conversion of trypsinogen to trypsin in mammalian systems; and/or (iii) enhancing the potency of the venom by also producing bioactive components (Shimek, 1975). We have discovered an unusual and unexpected gene product expressed in the salivary gland of the worm-hunting species *Conus pulicarius*, which may be consistent with the last possibility.

2. Material and methods

2.1. Specimen collection and RNA isolation

Specimens of *C. pulicarius* (Fig. 1) were collected at depths ranging from 0.5 to 1.0 m among the intertidal sea grass beds of Pago Bay, Guam, USA. Each specimen was dissected to isolate the venom duct and the salivary gland, which were immediately suspended in 1.0 mL RNAlater solution (Ambion, Austin, TX) and stored at −80 °C until used. The venom duct and salivary gland of a single specimen were each homogenized within their original microcentrifuge tube (1.6 mL) using 1.0 mL of TRIzol reagent and a hand-held Teflon pestle. Total RNA from each tissue was isolated by phase separation and column purification, according to the manufacturer's recommendations (TRIzol® Plus RNA Purification System; Invitrogen Corp., Carlsbad, CA).

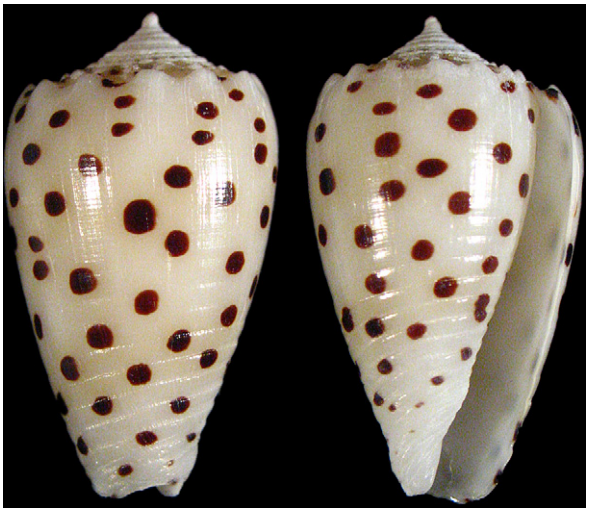

Fig. 1. A picture of the shell of one of the actual *Conus pulicarius* specimens collected from Pago Bay, Guam. *C. pulicarius* is a vermivorous cone snail that inhabits shallow coastal waters throughout the Indo-Pacific.

2.2. Preparation and sequencing of cDNA clones

First-strand cDNA was prepared from 2 μg of either salivary gland or venom duct total RNA, followed by second strand synthesis and 20 additional amplification cycles, which were performed by long-distance polymerase chain reaction (LD-PCR; Creator™ SMART™ cDNA Library Construction Kit; Clontech Laboratories, Inc., Palo Alto, CA) using an MJ Research PTC-200 Peltier Thermal Cycler. The resulting PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen Sciences, Germantown, MD) following the manufacturer's standard protocol. The resulting venom duct cDNA library was submitted for 454-Sequencing (Courtesy of Roche Colorado Corporation, Boulder, CO); due to the inherent difficulties in resolving the redundancies among transcripts found within this *C. pulicarius* venom duct, the results from this work will be presented at a later date. For the salivary gland cDNA pool, a second amplification (5 cycles), using tailed PCR primers, was performed on the PCR products. The resulting PCR products were size-fractionated using the CHROMA SPIN-400 DEPC columns supplied with the cDNA library construction kit. Eluted DNA was cloned into the pNEB206A vector and the resulting products transformed into chemically competent NEB 5-alpha cells, using the USER™ Friendly Cloning Kit (New England Biolabs, Inc., Ipswich, MD). The nucleic acid sequences of the resulting clones were determined using an ABI automated sequencer (Courtesy of the DNA Sequencing and Genomics Core Facility, University of Utah). The sequences of these independent clones were compared to identify PCR errors, and the resulting sequences obtained have been submitted to GenBank.

2.3. PCR-based screening for salivary gland α-conotoxins

A PCR assay capable of identifying salivary gland α-conopeptides from a *C. pulicarius* venom duct cDNA library was based on the PCR primers *SG-ALPHA-F* (5′-GGT CTG ACG TGA ACA CGA CAA TG-3′) and *SG-ALPHA-R* (5′-GCA ACC GTC TGA AGT GGA GGG CG-3′). The sequence used to design primers for amplifying standard α-conopeptide transcripts has been previously published (Santos et al., 2004). Transcripts encoding α-conotoxins were amplified from the cDNA pools by performing Nested-PCR using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and 40 cycles of amplification (annealing temperature = 55 °C; extension temperature = 68 °C) in an MJ Research PTC-200 Peltier Thermal Cycler. If a band was visible after