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High molecular weight components of the injected venom of fish-hunting cone snails target the vascular system

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ABSTRACT

The venom of marine cone snails is a rich source of pharmacotherapeutic compounds with striking target specificity and functional diversity. Small, disulfide-rich peptide toxins are the most well characterized active compounds in cone snail venom. However, reports on the presence of larger polypeptides have recently emerged. The majority of these studies have focused on the content of the dissected venom gland rather than the injected venom itself. Recent breakthroughs in the sensitivity of protein and nucleotide sequencing techniques allow for the exploration of the proteomic diversity of injected venom. Using mass spectrometric analysis of injected venoms of the two fish-hunting cone snails *Conus purpurascens* and *Conus ermineus*, we demonstrate the presence of angiotensin-converting enzyme-1 (ACE-1) and endothelin converting enzyme-1 (ECE-1), metalloproteases that activate potent vasoconstrictive peptides. ACE activity was confirmed in the venom of *C. purpurascens* and was significantly reduced in venom preincubated with the ACE inhibitor captopril. Reverse-transcription PCR demonstrated that these enzymes are expressed in the venom glands of other cone snail species with different prey preferences. These findings strongly suggest that cone snails employ compounds that cause disruption of cardiovascular function as part of their complex envenomation strategy, leading to the enhancement of neurotropic peptide toxin activity.

Biological significance
To our knowledge, this is the first study to show the presence of ACE and ECE in the venom of cone snails. Identification of these vasoactive peptide-releasing proteases in the injected venoms of two fish-hunting cone snails highlights their role in envenomation and enhances our understanding of the complex hunting strategies utilized by these marine predators. Our findings on the expression of these enzymes in other cone snail species

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suggests an important biological role of ACE and ECE in these animals and points towards recruitment into venom from general physiological processes.

1. Introduction

Cone snails have evolved remarkable strategies to capture prey, deter competitors, and defend against predators using a complex venomous cocktail. The venom is produced in a dedicated venom gland (also known as the venom duct) and delivered through a specialized injection apparatus consisting of a hollow harpoon-like radula tooth. Upon injection, the venom cocktail causes rapid immobilization of the prey. The injected venom contains a multitude of biologically active compounds that are predominantly targeted at the prey's nervous system. The majority of these compounds are conotoxins, an extremely diverse class of small disulfide-rich peptides. Conotoxins target receptors and ion channels, including different subtypes of ligand-gated nicotinic acetylcholine receptors [1], voltage-gated sodium [2], calcium [3,4], and potassium channels [5] and G-protein coupled receptors such as the vasopressin [6] and adrenergic receptors [7].

Studies of cone snail venom content have used both venom extruded from dissected venom glands and injected (milked) venom. Extruding venom requires sacrificing specimens and likely results in contamination by compounds released from glandular epithelial cells during sample preparation. Most studies of cone snail venom have focused on the discovery of small peptides; however, recent enhancements in nucleic acid and protein sequencing techniques have started to reveal the presence of larger, functionally diverse polypeptides. These include phospholipases of the A_2 family (PLA_2) [8,9], proteases of the cysteine-rich secretory protein (CRISP) family [8,10,11], pore-forming actinoporin-like proteins [8] and hyaluronidases, enzymes that cleave glycosidic bonds of the extracellular matrix [8,12]. The latter two were sequenced directly from the injected venom [8,12] indicating a role in the envenomation process rather than being released from glandular epithelial cells during sample preparation.

The presence of these diverse proteins indicates that, besides exerting potent neurotoxic effects, cone snail venom also causes tissue degradation and cell lysis via the action of PLA_2, proteases, actinoporins and hyaluronidases. Other molecules that are commonly found in animal venoms such as protease inhibitors and inhibitors of coagulation factors have not been demonstrated in injected cone snail venom although evidence for their expression in the venom gland is emerging [13]. In light of the sophisticated hunting strategies used by cone snails, it is likely that these and/or similar mechanisms also exist.

Using highly sensitive proteomic sequencing techniques combined with analysis of recently published transcriptome data, we have identified a new class of cone snail venom components. The identification of ACE-1 and ECE-1, enzymes that release potent vasoconstriction-inducing peptides, strongly suggests that the venom of cone snails comprises compounds for the disruption of cardiovascular function. Their discovery represents another milestone in understanding the complex and multidimensional hunting strategies utilized by these marine predators.

2. Materials and methods

2.1. Specimen collection

Specimens of *Conus purpurascens* were intertidally collected from several locations off the Pacific Coast of Costa Rica. Specimens of *Conus ermineus* were collected by nocturnal SCUBA immersions at 15-30 m depths off the reef systems of Palm Beach County, Florida, USA.

2.2. Extraction of injected venom

Injected venoms of *C. purpurascens* and *C. ermineus* were extracted according to the procedure of Hopkins et al. [14] with modifications [15]. Venoms were lyophilized and stored at −80 °C until further analyses.

2.3. RNA extraction, preparation of cDNA and reverse-transcription PCR

Venom glands were dissected from live specimens of *C. purpurascens*, *Conus victoriae* and *Conus novaehollandiae*, immediately snap-frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted and cDNA was prepared as previously described [16]. Oligonucleotides were designed based on sequence information made available in the recently published venom gland transcriptome of *Conus bullatus* [17]. The following oligonucleotide sequences were used in this study (5′-3′): sense ACE-1: CAG TTC CAG TTC CAC CAG GC; antisense ACE-1: GTT CTB YTT CWC CAG CCA; sense ECE-1: AGT WYG ACR AGG ABG GSA AC; antisense ECE-1: GCG ATG TTC TCM CYC AGR GT; sense ferritin: TTC ATG CCA GTT ATT GCT AC; antisense ferritin: CAT CCC GAT CAG GTT TCT where the mixed base codes are R(AG), Y(CT), M(AC), S(GC), W(AT), and B(GCT). PCR amplicons were analyzed by gel electrophoresis, cloned into pGEM-T plasmid vectors (Promega) and subsequently sequenced as previously described [18]. Nucleotide sequences were translated into the predicted amino acid residues and comparative alignment was performed using MAFFT auto sequence alignment by means of local pairwise alignment information [19].

2.4. Protein labelling and gel electrophoresis

Lyophilized venom obtained from a single extraction was reconstituted in 30 μL of destreak rehydration solution, pH 8.5 (GE Healthcare) at a final concentration of 1.5 μg/μL. For fluorescent labelling, venom samples were incubated with the fluorescent dye Cy5 (GE Healthcare) at 10 pmol/μL for 30 min on ice. Labelling was terminated with 1 μL of 10 mM L-lysine. Venom proteins were reduced by adding dithiothreitol to a final concentration of 20 mM. For gel electrophoresis, 15 μg of protein in rehydration solution was separated on 4–12% Bis-Tris gradient gels (NuPage, Invitrogen) for 30 min at 200 V in MES buffer. To visualize fluorescently labelled proteins, gels were