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Comparative analysis of proteases in the injected and dissected venom of cone snail species

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ABSTRACT

The venom of cone snails has been the subject of intense studies because it contains small neuroactive peptides of therapeutic value. However, much less is known about their larger proteins counterparts and their role in prey envenomation. Here, we analyzed the proteolytic enzymes in the injected venom of *Conus purpurascens* and *Conus ermineus* (piscivorous), and the dissected venom of *C. purpurascens*, *Conus marmoreus* (molluscivorous) and *Conus virgo* (vermivorous). Zymograms show that all venom samples displayed proteolytic activity on gelatin. However, the electrophoresis patterns and sizes of the proteases varied considerably among these four species. The protease distribution also varied dramatically between the injected and dissected venom of *C. purpurascens*. Protease inhibitors demonstrated that serine and metalloproteases are responsible for the gelatinolytic activity. We found fibrinogenolytic activity in the injected venom of *C. ermineus* suggesting that this venom might have effects on the hemostatic system of the prey. Remarkable differences in protein and protease expression were found in different sections of the venom duct, indicating that these components are related to the storage granules and that they participate in venom biosynthesis. Consequently, different conoproteases play major roles in venom processing and prey envenomation.

1. Introduction

The venom of predatory animals, such as snakes, spiders, scorpions, sea anemones and cone snails, contain an extraordinary array of peptides and proteins that target specific ion channels, neuronal receptors, and disrupt membranes and tissues as part of their envenomation strategy for predation. The venom of cone snails, a genus (*Conus* sp.) of marine gastropods that includes more than 700 extant species, varies dramatically from species to species. *Conus* species show prey preferences, as some prey upon fish (piscivorous), others on mollusks (molluscivorous) and the vast majority of cone snails feed on worms (vermivorous). There are enormous differences in the physiology, behavior and venom composition among cone snails depending on their prey preferences.

The venom apparatus comprises a venom duct, where the venom is synthesized and stored; a venom bulb, which propels the venom out from the duct; and a harpoon, which serves to inject the venom into the prey. Cone snails have a distensible proboscis and when it senses the prey, a single harpoon tooth is transferred from a radular sac to the lumen of the proboscis and therefore propelled for an efficient delivery of the venom (Olivera, 1997). The venom components are stored in the duct by microscopic granules suspended in a fluid; up to 60% of the molecular components in venom are associated with the granular fraction (Jimenez et al., 1983; Marshall et al., 2002). Most studies use the venom extracted out the dissected ducts (dissected

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venom); however, the actual venom injected into the prey (injected venom) can be obtained for species that prey upon fish (Hopkins et al., 1995).

While the small peptidic components of the venom (conopeptides) have been the focus of intense studies (Halai and Craik, 2009; Han et al., 2008; Lewis, 2009), the larger protein components of *Conus* venom (conoproteins) have been less comprehensively studied. The dissected *Conus* venom is known to contain enzymes, such as acetylcholinesterase, proteases, phosphodiesterases (Pali et al., 1979), toxin precursor molecules and other undescribed proteins (Marsh, 1970). Gel electrophoresis analyses indicated that there are proteins that vary widely in molecular weight in the dissected venom of several cone snail species (Cruz et al., 1976). More recent proteomic studies have revealed a wealth of proteins (more than 150 identifiable proteins per species) in the venom ducts of *Conus novaehollandiae* and *Conus victoriae* (Safavi-Hemami et al., 2011). 66 proteins were identified in the dissected venom of *Conus consors*; surprisingly, in the same study, only two proteins were identified in the injected venom of this species (Leonardi et al., 2012). Just as in other venomous animals, larger molecules present in *Conus* venom can perform a variety of functions, acting as neurotoxins, carrier proteins, or degradative enzymes such as proteases, hyaluronidases and lipases, and in toxin processing (Terlau and Olivera, 2004).

Proteases are degradative enzymes that achieve cellular control of essential biological processes through a highly specific hydrolysis of peptide bonds. In animal venom, proteases exert physiological effects on the prey, such as tissue degradation, which results in more efficient venom diffusion (Lopez-Otin and Overall, 2002). Marsh has shown that proteases are present in the venom of several species of vermivorous cone snails (Marsh, 1971). A protease belonging to the cysteine-rich secretory protein (CRISP) was isolated from the dissected venom of *Conus textile* (Tex31) (Milne et al., 2003). Tex31 was shown to be capable of cleaving the conotoxin TxVIA from its precursor. However, proteins that belong to the CRISP family, like helothermines, are found in snake and lizard venom and block skeletal and cardiac ryanodine receptors specifically (Lopez-Otin and Overall, 2002; Mackessy, 2002). Proteomic analyses of the venom duct of *C. novaehollandiae* identified high abundance of kallikrein-like proteins (Safavi-Hemami et al., 2011). Kallikreins are serine proteases found in animal venom that facilitate the degradation of kinins and fibrinogens on the prey (Asgari et al., 2003). Other proteases were also found in the venom duct of *C. victoriae* (Safavi-Hemami et al., 2011), such as the aspartyl protease cathepsin-D-like and proline peptidases. Cathepsin D cleaves fibronectin and it breaks drown extracellular matrices (Benes et al., 2008). Proline peptidases are involved in several cellular processes not necessarily related to envenomation (Walter et al., 1980). Proteomics analysis of the dissected venom of *C. consors* revealed the presence CRISP proteases and M12A peptidases, a class of zinc metalloproteinases that share common features with serralysins, matrix metalloendopeptidases, and snake venom proteases (Bond and Beynon, 1995).

In contrast to the widely documented effects of common venomous animal bites or stings, which manifest as hemorrhage, necrosis and inflammatory reactions, the degradative effects of cone snail venom are largely undescribed. An early study of some vermivorous Conidae found that the venom produces degradative effects in mice, such as tissue necrosis and hemorrhage at the site of the injection (Endean and Rudkin, 1965). Some cone snail stings in humans could lead to subcutaneous abscesses, which can be accompanied by pain, paraesthesia, general malaise, and fever (Veraldi et al., 2011).

Here we describe the distribution of proteases in the injected venom of two piscivorous species, *Conus purpurascens* and *Conus ermineus* (Eastern Pacific and Western Atlantic species, respectively), and in the dissected venom of *Conus marmoreus* (an Indo Pacific molluscivorous species) and *Conus virgo* (an Indo Pacific vermivorous species). We compared the venom protease content among these cone snail species with different feeding preferences and between dissected and injected venom for *C. purpurascens*. We compared that metalloproteases and serine proteases not only participate in intrinsic venom biosynthesis but might also affect the hemostatic mechanism of the prey, as the injected venom from *C. ermineus* had fibrinogenolytic effects.

2. Materials and methods

2.1. Extraction of dissected venom

Live specimens of all cone snail species here used were kept in aquaria in our laboratory prior to their dissection. The specimens of *C. marmoreus* and *C. virgo* were collected at several locations off the Vanuatu archipelago. Specimens of *C. purpurascens* were collected from several locations off the Pacific Coast of Costa Rica. The animals were sacrificed by placing them in an ice bath. The venom ducts were dissected from 4 specimens of *C. marmoreus*, 2 specimens of *C. virgo* and 2 specimens of *C. purpurascens*. Injected venom was extracted from the specimens of *C. purpurascens* before their dissected venom extraction (see below). The ducts were separately homogenized using a Dounce homogenizer in 5 mM Tris–HCl pH 7.5, 8 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ at 4 °C. Whole extracts were centrifuged at 10,000 *g* for 20 min, at 4 °C, and the resulting pellets were washed three times and re-centrifuged under identical conditions. The supernatants were pooled, lyophilized, and stored at −80 °C until further use.

2.2. Extraction of injected venom

Extraction of injected venom samples of *C. purpurascens* and *C. ermineus* were carried out according to the procedure of Hopkins et al. (1995) with modifications (Moller and Mari, 2011). Specimens of *C. ermineus* were collected from several locations off the South East Florida coast and the corresponding injected venom was extracted as described above.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using NuPAGE-Novex precast 10–20% polyacrylamide gradient tris-glycine gels or 10% polyacrylamide tris-glycine gels. Electrophoretical