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Danger in the reef: Proteome, toxicity, and neutralization of the venom of the olive sea snake, *Aipysurus laevis*

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ABSTRACT

Four specimens of the olive sea snake, *Aipysurus laevis*, were collected off the coast of Western Australia, and the venom proteome was characterized and quantitatively estimated by RP-HPLC, SDS-PAGE, and MALDI-TOF-TOF analyses. *A. laevis* venom is remarkably simple and consists of phospholipases A_2 (71.2%), three-finger toxins (3FTx; 25.3%), cysteine-rich secretory proteins (CRISP; 2.5%), and traces of a complement control module protein (CCM; 0.2%). Using a Toxicity Score, the most lethal components were determined to be short neurotoxins. Whole venom had an intravenous LD_{50} of 0.07 mg/kg in mice and showed a high phospholipase A_2 activity, but no proteinase activity *in vitro*. Preclinical assessment of neutralization and ELISA immunoprofiling showed that BioCSL Sea Snake Antivenom was effective in cross-neutralizing *A. laevis* venom with an ED_{50} of 821 μg venom per mL antivenom, with a binding preference towards short neurotoxins, due to the high degree of conservation between short neurotoxins from *A. laevis* and *Enhydrina schistosa* venom. Our results point towards the possibility of developing recombinant antibodies or synthetic inhibitors against *A. laevis* venom due to its simplicity.

1. Introduction

The viviparous sea snakes are a diverse clade of more than 60 species that are phylogenetically nested within the front-fanged Australo-Melanesian terrestrial elapids (Hydrophiinae) (Rasmussen et al., 2011). They are highly aquatic and occupy most shallow-marine habitats throughout the tropical and subtropical Indo-West Pacific, yet are estimated to share a common ancestor dated at only 6–8 million years ago (Sanders et al., 2008; Lukoschek et al., 2012). The amphibious sea kraits (Hydrophiinae: Laticauda) represent an independently aquatic and earlier diverging lineage that is the sister to terrestrial and viviparous marine hydrophiines (Keogh, 1998; Scanlon and Lee, 2004; Sanders et al., 2008). Two major clades are recognized within the viviparous marine group: An 'Aipysurus' lineage comprising ten species found primarily in the Australo-Papuan region, and a 'Hydrophis' lineage containing at least 50 species distributed throughout the Indo-West Pacific (Rasmussen et al., 2011).

In the *Aipysurus* group, the olive sea snake, *Aipysurus laevis*, has a large muscular head and is the most robustly built and longest species recorded, reaching more than 170 cm in total length (Smith, 1926; Cogger, 1975). *A. laevis* has been recorded from Aru Archipelago and Kai Islands (Indonesia) in the west and from the northern coast of Australia and southern coast of New Guinea (Timor Sea and Arafura Sea) to New Caledonia in the east (Coral Sea) (Cogger, 1975; Ineich and Rasmussen, 1997; Sanders et al., 2014). *A. laevis* is found in shallow marine habitats – coral reefs as well as sandy, rocky, and mud-bottom habitats, and is often one of the most abundant species throughout its range (Cogger, 1975; Lukoschek et al., 2007; Sanders et al., 2014). It hunts primarily in crevices on the sea floor, and the following fish families have been found as prey items in *A. laevis*: Acanthuridae, Apogonidae, Carangidae, Clupeidae, Engraulidae, Labridae, Lutjanidae, Pempheridae, Pomacentridae, Scaridae, Scorpaenidae and Serranidae (McCosker, 1975; Voris and Voris, 1983). Fish eggs, crabs, shrimp

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and pelecypod (Limidae) have also been found in stomach content (McCosker, 1975; Voris and Voris, 1983).

During mating season *A. laevis* is more prone to defensive attacks than at other times of the year (Heatwole, 1975a,b). However, normally *A. laevis* will ignore a diver even if the diver approaches quite close (Heatwole, 1975a,b). *A. laevis* has up to at least 5 mm long fangs and the venom is known for being extremely toxic (Limpus, 1978; Minton, 1983; Mackessy and Tu, 1993; Greer, 1997). *A. laevis* is commonly caught as by-catch, and commercial trawler fishers and recreational fishers handling nets are therefore the typical bite victims of *A. laevis*.

The venoms of sea snakes, typically containing α-neurotoxins and phospholipases A_2 (PLA_2s), are known to be generally more potent than the venoms from terrestrial snakes in terms of lethality (Minton, 1983; Takasaki, 1998). In contrast to the latter, however, only few studies have been focused on determining the comprehensive composition of sea snake venoms by means of proteomic analyses, i.e. venomics. The venom of *A. laevis* has been shown to be neurotoxic, nephrotoxic, and myotoxic in mice, causing acute renal tubular degeneration, proliferative glomerulonephritis, local muscle degeneration, necrosis, enlarged spleen, inflammation, and lymphadenopathy (Zimmerman et al., 1992a, 1992b; Ryan and Yong, 1997, 2002). Regarding the venom components of *A. laevis*, a total of four short-chain neurotoxin isoforms with minor amino acid sequence variations (P19958, P19959, P19960, and P32879) and one PLA_2 (P08872) have been fully sequenced (Maeda and Tamiya, 1976; Ducancel et al., 1988, 1990). The short α-neurotoxins display a high affinity towards the acetylcholine receptor (Ishikawa et al., 1977), which is in agreement with the very low LD_{50} observed for the whole venom (Tamiya, 1973; Maeda and Tamiya, 1976). Toxicity of the venom has additionally been tested in different fish species, showing variations in responses (Berman, 1983; Zimmerman et al., 1990, 1992a, 1992b). It has been suggested that several components of the venom may act in a synergistic manner to potentiate toxic effects (Ryan and Yong, 1997). Finally, antivenoms raised against tiger snake (*Notechis scutatus*) or common sea snake (*Enhydrina schistosa*) venoms have been shown to have some cross-reactivity towards the venom of *A. laevis*, although the efficacies of these antivenoms are lower than against the venoms of homologous species (Baxter and Gallichio, 1974).

Aiming to further develop understanding of sea snake venoms and to expand knowledge of venom intra-species variability, this study presents the proteomic analysis of the venom of *A. laevis*, together with an assessment of variability in three different specimens, and of toxicity of all its main protein components in mice. In addition, the ability to cross-recognize and neutralize *A. laevis* venom was evaluated for two antivenoms against coral snakes and sea snakes.

2. Materials and methods

2.1. Snake venom

A. laevis venom was obtained from four specimens ("Mifisto", "Medusa", "His", and "Nessi") kept at the National Aquarium, Den Blå Planet, Denmark. All specimens were collected at night by Kate L. Sanders from a boat using spotlights and dip nets. The boat was operating at shallow water close to Broome, Australia. The venom, collected in the National Aquarium of Denmark, was immediately frozen, lyophilized, and kept at −20 °C. In order to assess individual variability, a small sample of venom from each snake was kept separated, while the remaining material was pooled.

2.2. Venom separation by reverse-phase HPLC and SDS-PAGE

The pooled venom of *A. laevis* was fractionated by sequential RP-HPLC and SDS-PAGE separation steps, following the 'snake venomics' analytical strategy (Calvete, 2011) under conditions described previously (Lomonte et al., 2014a,b). Venom load for the RP-HPLC step on C_{18} (4.6 × 250 mm column, 5 μm particle diameter; Teknokroma) was 2 mg. Protein fractions were monitored at 215 nm, manually collected, and dried by vacuum centrifugation. A sample of each RP-HPLC fraction was adequately diluted in either α-cyanohydroxycinnamic acid or sinapinic acid saturated solutions in 50% acetonitrile containing 0.1% trifluoroacetic acid, and spotted onto an Opti-TOF plate for MALDI-TOF mass spectrometry analysis in linear positive mode, using an AB4800-Plus Proteomics Analyzer (Applied Biosystems). The observed isotope-averaged masses in each fraction were reported as mean ± SD of four replicate determinations. Each RP-HPLC fraction was electrophoretically separated under reducing conditions. Resulting bands were stained with colloidal Coomassie blue G-250, and digitally recorded on a ChemiDoc® imager using ImageLab® software (Bio-Rad).

2.3. Protein identification by tandem mass spectrometry of tryptic peptides

Protein bands were excised from gels, destained with 50% acetonitrile in 25 mM ammonium bicarbonate, and then subjected to reduction (10 mM dithiothreitol), alkylation (50 mM iodoacetamide), and overnight in-gel digestion with sequencing grade trypsin (Sigma), in 50 mM ammonium bicarbonate at 37 °C. The resulting tryptic peptides were extracted with 50% acetonitrile containing 1% trifluoroacetic acid (TFA), and analyzed by MALDI-TOF-TOF in positive reflector mode, under conditions previously described (Lomonte et al., 2014a,b). In each run, CalMix® standards (ABSciex) spotted onto the same plate were used as external calibrants. Resulting spectra were searched against the UniProt/SwissProt database using ProteinPilot® v.4 and the Paragon® algorithm (ABSciex) for protein identification at ≥95% score confidence, or manually interpreted. Few peptide sequences with lower confidence scores were manually searched using BLAST (http://blast.ncbi.nlm.nih.gov) for protein similarity and assignment to protein families.

2.4. Relative protein abundance estimations

Areas of the RP-HPLC chromatographic peaks at 215 nm were integrated using ChemStation® (Agilent) in order to estimate relative protein abundances (Calvete, 2011). For peaks containing several electrophoretic bands, percentage distributions were assigned by densitometry, using ImageLab® (Bio-Rad).

2.5. Phospholipase A_2 and proteolytic enzyme activities

Enzymatic activities of *A. laevis* venom were tested comparatively with samples obtained from other elapid snakes (*Dendroaspis polylepis*, *Naja kaouthia*; obtained from Latoxan, France; and *Micrurus nigrocinctus*, obtained from Instituto Clodomiro Picado) or the viperid *Bothrops asper* (Instituto Clodomiro Picado). PLA_2 activity was assayed on the chromogenic 4-nitro-3-octanoyloxybenzoic acid (NOBA) synthetic substrate, as described (Lomonte et al., 2015). Venoms (20 μg, dissolved in 25 μL of 10 mM Tris, 10 mM $CaCl_2$, 0.1 M NaCl, pH 8.0, buffer) were mixed with 200 μL of the same buffer and 25 μL of NOBA to achieve a final substrate concentration of 0.32 mM. Plates were incubated for 60 min at 37 °C, and absorbance was recorded at 405 nm in a microplate reader. Proteolytic activity was determined on azocasein, according to Wang et al. (2004). Venoms (40 μg, dissolved in 50 mM Tris–HCl, 0.15 M NaCl, 5 mM $CaCl_2$ buffer, pH 8.0) were added to 100 μL of azocasein (10 mg/mL in the same buffer), and incubated for