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Profil D'expression des Protéines de Venin dans *Pterois volitans*: Implications pour la Détection de la Ciguatoxin

CHRISTIE WILCOX
University of Hawaii, Hawaii Institute of Marine Biology, PO Box 1346, Kaneohe, Hawaii 96744 USA.
*wilcoxcl@hawaii.edu.

ABSTRACT

The fish in the order Scorpaeniformes posses potent venoms that cause neuromuscular and cardiovascular symptoms through the activation of sodium channels, calcium influx into cells, and the release and depletion of acetylcholine from presynaptic neurons. Although these venom compounds have the potential to mimic ciguatoxin in detection bioassays, thus disrupting our ability to accurately test for ciguatera in venomous species, they are harmless to fish consumers. While it is know that the venom is present in the spines, no research has sought investigated if venom proteins exist elsewhere in the fish. Proteins were extracted from the spine, skin, muscle and liver tissues of fish using four buffers used by previous studies to extract ciguatoxin samples: phosphate-buffered saline, 70% methanol, 100% methanol and 100% acetone. Western blotting with stonefish antivenom was used to detect the presence of venom proteins in tissues from invasive *Pterois volitans*. Venom proteins were most highly expressed in the spines of the venomous Scorpaeniform species, with decreased but detectable expression in skin and muscle tissues. These proteins were detected strongly in both the saline and 70% methanol extracts, suggesting the possibility that these proteins or other venom compounds contaminate ciguatoxin tests. The next step of this research, clarifying the affect of venom toxins on ciguatera bioassays, will increase the accuracy of ciguatera detection in Scorpaeniform species, potentially expanding the fishery for invasive lionfishes as well as other commercially relevant Scorpaeniformes.

KEY WORDS: Lionfish, ciguatera, ciguatoxin, scorpaenitoxin, venom

INTRODUCTION

Ciguatera fish poisoning (CFP) is the most common marine poisoning worldwide with at least 50,000 cases reported every year (Ting and Brown 2001). The responsible agents are ciguatoxin and its close congeners (CTXs), colorless and odorless bioaccumulating lipophilic toxins produced by reef-associated dinoflagellates found globally in tropical and subtropical latitudes. Because the toxins are not inactivated through any normal means of fish preparation, detection of fish with high toxin concentration is the only way to prevent poisoning. Because CTX testing is expensive and time consuming, where CFP is endemic, large, predatory reef fish are avoided, reducing the number of fish available for sustenance and increasing fishing pressure on other species. Even a few cases of CFP can drastically alter the use of reef resources, and fish avoidance can have an adverse economic impact (Lewis 1986). In French Polynesia, for example, CFP costs over $1 million dollars annually in lost productivity due to illness and more than $1 million in lost earnings due to banned fish (Glaziou and Legrand 1994).

Although *P. volitans/miles* are only mid-sized predators (mesopredators), they are typically piscivorous, and so have the potential to be ciguatoxic. Their relatively long lifespan (decades) increases their bioaccumulation potential, and thus careful study of CTX prevalence in these species is warranted and necessary to ensure safe consumption. There have been no published peer-reviewed studies to date on the prevalence of CTXs in lionfishes, either in their native or invasive ranges. However, results from the 2012 Florida Sea Grant/FDA investigation concluded that among 194 fish tested, 42% showed detectable levels of CTXs and 26% were above the FDA's illness threshold of 0.1 parts per billion (Gill 2012). The method of testing was not reported. However, the venom ubiquitous to lionfish species may mimic ciguatoxin in bioassays, potentially causing false positives.

Clinically, ciguatera poisoning and lionfish envenomation generate overlapping symptoms, indicating that similar effects may be occurring at the cellular level. The predominant symptom of lionfish envenomation is intense, throbbing pain at the sting site, which may radiate from the site of injury and persist up to 12 hours (Halstead 1988, Trestrail and Mahasneh 1989). However, anesthesia, paresthesia, and hypesthesia have all been reported, and all are symptoms of ciguatera poisoning (Kasdan et al. 1987, Trestrail and Al-Mahasneh 1989, Kizer et al. 1985, Patel and Wells 1993). While systemic effects of envenomations are less common, they are similar to clinical presentations of CFP. These include headache, nausea, vomiting, abdominal pain, delirium, seizures, limb paralysis, hypertension and hypotension, respiratory distress, heart problems, muscle weakness, chills and death (Kasdan et al. 1987, Trestrail and Mahasneh 1989, Kizer et al. 1985). Intravenous introduction of scorpaeniform venom extracts in mice yield similar effects to injection of ciguatoxin, including ataxia, limb paralysis, muscle weakness, and death, with muscular effects more pronounced for lionfish venom than

stonefish venom (Saunders and Taylor 1959).

Most methods of ciguatera detection depend on physiological effects in test animals, using *in vitro* bioassays to determine presence and concentration of CTXs. Intraperitoneal injection of mice with crude fish extracts has been used by the Hawaii Department of Health to detect CTX (Hokama 2004), with key indicators of toxicity being weakness, paralysis and death. However, these effects are also seen with intraperitoneal injections of scorpaeniform extracts (Khoo et al. 1992, Saunders 1960, Saunders and Taylor 1959, Khoo 2002, Shiomi et al. 1989). In guinea pig atria, both CTX and stonustoxin cause negative inotropy associated with cell depolarization and calcium overload (Lewis 1988, Austin et al. 1965). CTX is also a highly potent sodium channel activator, and a number of assays, like the rapid hemolysis assay and the neuroblastoma assay (Shimojo and Iwaoka 2000), assess sodium channel activation as an indicator of CTX. Yet stonustoxin from scorpaeniform fishes has also been shown to activate sodium channels, and like CTX, activating effects are blocked by sodium channel blockers such as tetrodotoxin (Hopkins et al. 1996).

The isolation of similar protein toxins across distant lineages (Poh et al. 1991, Ghadessy et al. 1996, Garnier et al. 1995, Garnier et al. 1997, Ueda et al. 2006, Kreger 1991, Kiriake and Shiomi 2011, Andrich et al. 2010) demonstrates that a unique toxin family (scorpaenitoxins) is highly conserved in this taxonomic group. Unlike CTX, scorpaenitoxins are readily degraded when heated or ingested. Thus while scorpaenitoxins might throw off a ciguatoxin test, they pose no threat to the consumer. Based on the high degree of similarity in the effects of CTX and scorpaeniform venoms, it is possible that bioassays for CTX are inaccurate in scorpaeniform species. The production of venom components could explain the putative prevalence of CTX in invasive lionfishes despite the complete lack of poisoning incidents. If this effect occurs, then it has likely gone undocumented because there are few commonly consumed ciguatoxic fish that might produce such toxins. These preliminary results suggests that scorpaenitoxins (a) are present in the tissues commonly used for ciguatera testing, and (b) can be detected after extraction protocols commonly used in ciguatera testing. These data indicate caution and skepticism when interpreting results from ciguatera testing in scorpaeniform fishes, including invasive lionfishes. The ultimate solution is more reliable and specific testing for ciguatera, allowing for improved management of these highly invasive species.

MATERIALS AND METHODS

Relative scorpaenitoxin levels in each sample were analyzed using a western blotting protocol as employed by previous scorpaenitoxin investigations (e.g. Andrich et al. 2010). Studies have shown that scorpaenitoxins from distantly related fishes, including lionfish, scorpionfish and soldierfish, all cross-react with stonefish antivenin, (SFAV) (Shiomi et al. 1989, Church and Hodgson 2001, Church and Hodgson 2003, Andrich et al. 2010), with scorpaenitoxin subunits around 75 kDa in size. Spine, skin, muscle and liver tissues were extracted using four ciguatoxin protocols: phosphate-buffered saline (PBS), 70% methanol, 100% methanol and 100% acetone. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to separate venom proteins by size, using the method developed by Laemmli (1970). For immunoblotting, proteins were electrotransferred onto a PVDF membrane, probed with SFAV diluted in PBS (1:500) and resolved using the HRP-linked anti-horse IgG and the Clarity Western ECL Substrate (Bio-Rad). Blots were visualized using GeneSnap (SynGene), with relative expression levels calculated using GeneTools (SynGene).

RESULTS AND DISCUSSION

As analyzed by immunoblotting, the antiserum reacted to a number of proteins ranging in size, though the strong reactivity by two proteins roughly 75 kDa in size is consistent with the alpha and beta subunits of PvTx identified by Kirikake and Shiomi (2011) (Figure 1A). These scorpaenitoxin bands were strongest in spine tissues (67.2 ± 46.3 times more optically dense than in liver tissues), but were also present in skin and muscle tissues at around $1/10^{th}$ the concentration (Figure 1C). Intact scorpaenitoxins of ~150 kDa were also detected in spine, muscle and skin (Figure 1B). Though scorpaenitoxins were best isolated using a saline extraction buffer, they were also detected in 70% methanol extractions (Figure 1D, E). 100% acetone extractions—commonly used in CTX analyses—also contained detectable levels of scorpaenitoxins, albeit at much lower levels. No scorpaenitoxins could be detected in the methanol extracts.

Our data indicate that while scorpaenitoxin venom components are highly concentrated in the spines of lionfishes responsible for the Atlantic invasion (*Pterois volitans/miles*), they can also be found throughout the body, including tissues commonly tested for the presence of ciguatoxin. Given that scorpaenitoxins and ciguatoxin cause similar biochemical reactions, it is possible that standard ciguatera tests of lionfish provide false positives. If so, lionfish that are safe to consume would be rejected by fisheries and consumers as being ciguatoxic, thereby inhibiting an management plan for mitigating impact of the invasion (Morris 2012). A simple solution is to cook a lionfish before conducting a ciguatera test, which denatures scorpaenitoxins, leaving only ciguatoxin, if present.

The lipophilic nature of scorpaenitoxins further contributes to concerns about contamination of ciguatera tests. Though methanol- and acetone-based extraction methods are designed to extract lipids, it is well established that other highly lipophilic compounds including proteins can be solubilized in these organic solvents (Erickson 1993). The presence of scorpaenitoxins in 100% acetone extracts is particularly troubling, as this is a common first

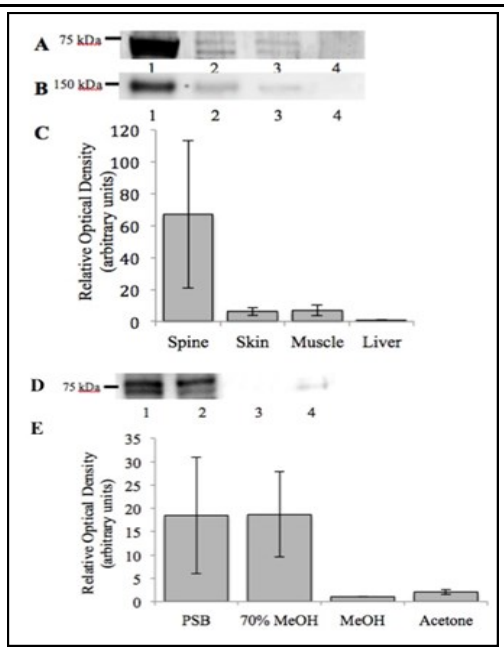

Figure 1. Relative scorpaenitoxin content in different tissues from invasive lionfish *Pterois volitans/miles*. A) Representative western blot after denaturing SDS-PAGE showing the detection of the alpha and beta subunits of PvTx in the various tissue types. Lanes: 1, spine; 2, skin; 3, muscle; 4, liver B) Representative western blot after Native-PAGE showing the detection of complete PvTx in the various tissue types. Lanes: 1, spine; 2, skin; 3, muscle; 4, liver. C) Relative scorpaenitoxin levels across tissue types. Values represent mean densitometry values of protein bands (± 1 standard error of the mean) detected by western blotting of SDS-PAGE; values represent band intensities standardized by the liver sample from each fish, which we use as a reference; n=4 for each bar D,E) Relative scorpaenitoxin content in different extraction methods from invasive lionfish *Pterois volitans/miles*. D) Representative western blot showing the detection of the alpha and beta subunits of PvTx in the various extraction methods. Lanes: 1, PBS buffer; 2, 70% Methanol; 3, 100% Methanol; 4, 100% Acetone. E) Relative scorpaenitoxin levels across extraction methods. Values represent mean densitometry values of protein bands (± 1 standard error of the mean) detected by western blotting; values represent band intensities standardized by the methanol sample, which we use as a reference; n=3 for each bar.

step for CTX detection. These results stress the importance of clean-up protocols and multiple extraction steps to ensure the purity of any samples tested for CTX. Again, the best precaution is to cook a lionfish before testing for the presence of CTX.

Though scorpaenitoxins are the most lethal component of scorpaeniform venoms, they are not the only toxic constituents that may play a role in the *in vitro* similarities with ciguatoxins. Venoms are usually complex chemical cocktails with multiple compounds contributing to activity (Casewell et al. 2012). Biologically active peptides have been isolated from multiple scorpaeniform fishes. Juzans et al. (1995) isolated a peptide from *Synanceia trachynis* that, like CTX, causes spontaneous release and depletion of acetylcholine from motor nerve terminals. Balasubashini et al. (2006) isolated an antiproliferative peptide (7.6 kDa) from *P. volitans* venom. In *P. volitans*, several proteins ranging in size cross react with stonefish antivenom, possibly indicating venomous use (data not shown). In other investigations of lionfish venom, proteolytic enzymes weighing roughly 45 kDa have been detected though not purified (Balasubashini et al. 2006), and other proteins weighing 29 kDa, 66 kDa, 97 kDa and 116 kDa have been separated using SDS-PAGE, though their functions are unknown (Choromanski 1985). Non-proteinaceous components have also been found in all scorpaeniform venoms examined. There is also strong evidence for the presence of neurotransmitters in the venoms of lionfish (Cohen and Olek 1989; Church and Hodgson 2002) and other Scorpaeniformes (Garnier et al. 1996), including acetylcholine and noradrenaline. Though very little investigation has focused on lipid toxins in these species, Nair et al. (1985) isolated an unknown lipophilic ichthyotoxin from *P. volitans* spines.

The detection of all of these components in different tissues and extraction methods was outside the scope of this work, but it is important to remember that with our limited knowledge of the relative contribution of venom components to in vitro activities, any of these could cause false positives in CTX activity assays. Some, like the unidentified toxin isolated by Nair et al. (1985), may be even more likely to survive lipid-specific extraction methods. Thus further research into the diversity of toxins in lionfish tissues is necessary to completely understand the potential for contamination of CTX assays. Though these data do not provide conclusive evidence of venom contamination, they provide enough support for the re-evaluation of our ciguatera testing methods in lionfish and other venomous species. Certainly caution is indicated in interpreting results from CTX bioassays of lionfish populations. While there is likely no doubt that lionfish in areas with high levels of ciguatera prevalence possess the same potential danger as similar mesopredators, there is no evidence that lionfish in ciguatera-free areas are a threat to public health.

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