PACIFIC-CIGUATOXINS (P-CTXS) IN CORAL REEF FISHES: TOXIN PURIFICATION, ANALYTICAL METHOD VALIDATION AND TROPHODYNAMICS IN MARINE FOOD WEB

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SEPTEMBER 2012
Pacific-Ciguatoxins (P-CTXs) in Coral Reef Fishes: Toxin Purification, Analytical Method Validation and Trophodynamics in Marine Food Web
珊瑚魚中太平洋雪卡毒素的純化、分析方法驗證及其在海洋食物網的營養級轉移

Submitted to
Department of Biology and Chemistry
生物及化學系
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
哲學博士學位

by

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September 2012
二零一二年九月
Abstract

Ciguatera fish poisoning (CFP) is a non-bacterial food-borne illness that results in gastrointestinal, neurological and cardiovascular disorders. Due to the increase in international trade of coral reef fishes, CFP is no longer restricted to endemic regions and has become a global health problem, affecting more than 50,000 people annually. Ciguatoxins (CTXs) are the causative agents of CFP, and more than 20 CTXs have been identified to date. Generally, CTXs have been classified into three groups: Pacific-CTXs (P-CTXs), Caribbean-CTXs (C-CTXs) and Indian-CTXs (I-CTXs). Among these toxins, P-CTX-1 has been suggested to be the most toxic CTX, causing CFP at levels equal to or greater than 0.1 ng/g in muscle of carnivorous fishes. Because of their lipophilicity, these toxins tend to accumulate in fish muscle and viscera. CTXs are stable molecules that resist extreme temperature and mildly acidic and alkaline conditions, and therefore coral reef fishes remain toxic after cooking. Ciguateric fishes are difficult to be identified because of their normal appearance, taste and smell, and therefore a reliable, accurate and rapid detection method for CTXs is required to protect human health. The objective of the present study was to isolate and purify P-CTX-1 from viscera of moray eels for use as authentic standard and to develop analytical methods to identify and quantify P-CTXs in fish muscle and fish blood that can be used to screen coral reef fishes in the marketplace. The dynamics of CTXs in a marine food web were also investigated so as to understand the sources and fates of these toxins. The results of this study provide important information for future risk assessment and
fisheries management related to the control of CFP.

Several analytical methods for P-CTX detection in fishes have been developed over the last decade, but a lack of analytical CTX standards has hindered the further development of rapid, reliable and robust screening methods for ciguateric fishes. To overcome this constraint, the first objective of the present study was to isolate and purify P-CTX-1 as an authentic standard to develop a P-CTX quantification method in fishes. Using 5.74 kg of viscera of undulated moray eels (*Gymnothorax undulatus*) and yellow-edged moray eels (*G. flavimarginatus*) collected from a CFP-endemic region in the Republic of Kiribati, 40 µg of P-CTX-1 was isolated and purified. The purity of the P-CTX-1 was confirmed and determined to be ≥ 95% using high-performance liquid chromatography coupled with a UV detector (HPLC-UV) monitored at 215 nm. The UV profile and mass spectrum of the purified P-CTX-1 were found to be comparable to those of a P-CTX-1 standard, indicating its suitability for use in analytical method development.

Screening of ciguatoxic fishes in the marketplace and food web analysis of CTXs requires analysis of many different marine species, but matrix recoveries reported for the previously established analytical methods for CTX detection were highly variable among species. Therefore, a new and more reliable method was developed for use in CTX quantification across a marine food web. The establishment and validation of an analytical method to quantify P-CTX-1 was
carried out initially in fish muscle using accelerated solvent extraction (ASE) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Methanol was chosen to be the extraction solvent. The optimized ASE conditions were determined to be two extraction cycles, 5 minutes of static time, 75°C, 1500 psi with 60% flush volume and 100 second of purge time. The present method is robust with matrix spike recoveries of P-CTX-1 in muscle of fifteen species of coral reef fish ranging from 66% − 85%, with the exception of two herbivorous fishes, lined surgeonfish (*Acanthurus lineatus*) and blue-barred parrotfish (*Scarus ghobban*), that showed recoveries of 49% and 51%, respectively. Although ion suppression occurred during LC-MS/MS analysis, the present method provided better P-CTX-1 recovery in fish muscle than a previously published method and P-CTX-1 levels determined using ASE-LC-MS/MS were well-correlated with ciguatoxicity measured using the *in vitro* mouse neuroblastoma assay (MNA), a standard method for CTX detection. The whole analytical process can be conducted within 8 hours, providing a rapid method for the determination of ciguateric fish in the marketplace. The limit of quantification (LOQ) of this method was evaluated to be 0.01 ng/g fish muscle which is the level considered to be safe for human consumption.

In addition to P-CTX-1, analogues of P-CTX-2 and P-CTX-3 were also identified in the fish muscle extracts using the current ASE-LC-MS/MS method. These findings confirmed the importance of the development of an analytical method to simultaneously determine the
concentrations of P-CTXs in coral reef fishes. However, the use of fish muscle for ciguateric fish screening may harm coral reef fishes, and matrix effects in fish muscle extracts were found to be significant. Therefore, the potential use of blood as a non-destructive indicator to estimate P-CTX levels in consumable tissue was investigated. A simultaneous analytical method for P-CTX-1, P-CTX-2 and P-CTX-3 in whole blood of coral reef fishes using sonication and LC-MS/MS for extraction and quantification was developed. Using acetonitrile with a solvent-to-blood volume ratio of 3:1, 1 – 5 mL fish blood can be used for P-CTX quantification. The optimal extraction efficiency (74 – 103%) of P-CTXs in fish blood was achieved when three extraction cycles of sonication, protein precipitation and centrifugation were employed. Matrix effects in fish blood extracts were found to be less significant than those in the muscle extracts; responses of P-CTX-1, P-CTX-2 and P-CTX-3 were enhanced or suppressed by 6 – 26% in fish blood extract. All three P-CTXs were detectable in whole blood of wild-caught giant moray (G javanicus), yellow-edged moray and blue-spotted grouper (Cephalopholis argus) collected from a CFP-endemic region, with matrix spike recoveries ranging from 74 – 86% (RSD ≤ 18%) and 96 – 111% (RSD ≤ 14%), respectively. Total P-CTX levels in whole blood samples generally conformed to the order of giant moray > yellow-edged moray > blue-spotted grouper, a trend similar to that found in muscle of these fishes. The similar patterns of P-CTX composition profiles, together with significant positive relationships between P-CTX-1 (n = 14; \( r^2 = 0.370; p = 0.021 \)) and total ciguatoxicity (n = 14; \( r^2 = 0.373; p = 0.020 \)) in blood and muscle of the wild-
caught moray eels demonstrated that P-CTX levels in fish blood have the potential to be used to extrapolate those in consumable fish muscle, supporting the use of whole blood for ciguateric fish screening. Based on the available threshold level in fish muscle (0.01 ng/g) for the prevention of acute induction of CFP, the threshold level for CFP in fish blood was estimated based on the relationship between total ciguatoxicity in fish blood and muscle and calculated to be 3.48 pg/mL, a level which was two times higher than the LOQ of the present method (1.75 pg/mL) when 4 mL fish blood was used for the analysis. This method is therefore valid for ciguateric moray eel screening but further investigations on P-CTX pharmacokinetics in coral reef fishes may help to reinforce the relationship between P-CTX concentrations in fish blood and muscle in different coral reef fish species.

Toxin transfer and transformation through the marine food chain has been suggested as the source of CTXs and thus the cause of CFP. According to this hypothesis, CTXs are biotransformation products of gambiertoxtins (GTXs) that are produced by benthic dinoflagellates such as *Gambierdiscus toxicus*. Herbivorous fishes grazing on macroalgae also ingest the associated epiphytic dinoflagellates and their toxins, and these fishes are then in turn preyed upon by carnivorous fishes; the fishes oxidize GTXs to CTXs, which then bioaccumulate and biomagnify up the food chain. Although this hypothesis was proposed in 1958, the dynamics of CTXs in marine food web are poorly understood. To confirm this hypothesis, a comprehensive
food web study was carried out and samples of algae (including green algae, encrusting red algae and red turf algae), sedimentary organic matter (SOM), organic matter on dead coral, invertebrates (including lobsters, crabs, shrimp and octopi), and fishes (including herbivores, omnivores and carnivores) were collected from two ciguatoxic sites and a reference site in Marakei Island of the Republic of Kiribati in June 2009 and May 2011.

Isotopic mixing model revealed that biofilm was an important food source for herbivorous surgeonfish (mean: 16.4%, 5 – 95% credibility: 3.62 – 28.3%) and parrotfish (29.9%, 16.3 – 44.5) collected from the ciguatoxic sites, whereas those fishes collected in the reference site had a relatively well-mixed diet. The difference in the relative contribution of biofilm to the diet of herbivorous fishes indicated that biofilm could be an important source of P-CTXs to grazers in the ciguatoxic sites. Angelfish was the major prey of blue-spotted grouper (28.5%, 8.39 – 47.6%) and yellow-edged moray (20.5%, 4.64 – 35.5%), whereas giant moray mainly fed on surgeonfish (herbivorous: 14.7%, 2.25 – 26.1%; omnivorous: 11.8%, 1.44 – 20.9%) and parrotfish (14.2%, 0.8 – 26.9%) in the ciguatoxic sites. At the ciguatoxic sites, the dominance of surgeonfish and parrotfish to the diet showed the following trend: blue-spotted grouper < yellow-edged moray < giant moray, and this was in accordance with the total P-CTX concentrations measured in these predators. This indicated the importance of surgeonfish and parrotfish as the key vectors to link CTX-producing agents to secondary consumers.
Generally, ciguatoxic fishes were restricted to the studied ciguatoxic area in Marakei. Total P-CTX concentrations of blue-spotted grouper, giant moray and yellow-edged moray collected from the studied ciguatoxic regions were 85-fold, 93-fold and 1240-fold higher than those collected from the reference site. The restriction of ciguatoxic fishes indicated that the toxins were not endogenous, but arose from unique environmental conditions in the studied area. P-CTX-1, P-CTX-2 and P-CTX-3 were detected in a lobster, an octopus, six species of herbivorous fishes, eight species of omnivorous fishes and 28 species of carnivorous fishes. Porcupinefish (*Diodon hystrix* and *Diodon liturosus*) occupied comparable trophic levels to moray eels, but their total P-CTX concentrations were significantly lower than those in moray eels. Also, porcupinefish and pufferfish (*Arothron nigropunctatus*) were of the genus *Tetraodontiformes* spp. Yet, only P-CTX-1 was detected in the carnivorous porcupinefish, but P-CTX-1, P-CTX-2 and P-CTX-3 were co-existed in the omnivorous pufferfish. These may be due to their dietary preferences in which porcupinefish was crustacean-feeder and/or mollusk-feeder, whereas pufferfish and moray eel mainly fed on primary sources and fishes, respectively. These results indicated diet as a crucial factor determining P-CTX concentrations and composition in fishes. In contrast, correlation analysis revealed that body size (in terms of total length and body weight) was only significantly correlated with P-CTX levels in giant moray. These significant relationships were only occasionally observed in yellow-edged moray and absent in blue-spotted grouper. In addition, significant correlations were not necessarily found between P-CTX levels,
trophic levels and lipid content of these fishes. This implied that body size, trophic level and lipid weight may not be the determinants governing the P-CTX levels in these fishes.

Of the measured P-CTXs, P-CTX-2 was usually the predominant P-CTX, and the ratios of P-CTX-1:P-CTX-2:P-CTX-3 were 11:57:30 and 14:61:25 in ciguatoxic herbivorous and omnivorous fishes, respectively. In contrast, carnivorous fishes collected in the ciguatoxic site exhibited diverse P-CTX composition profiles and the mean P-CTX-1:P-CTX-2:P-CTX-3 ratio of CTX-containing carnivorous fishes was 50:38:13. The diverse composition profiles of P-CTXs in carnivorous fishes may have been attributed to the interspecific variations of the fishes, in terms of their diets and metabolism such as rate of uptake, biotransformation, bioaccumulation and elimination of P-CTXs.

Invertebrates such as giant clams and shrimp have been suggested to be vectors to transfer CTXs in the food web. In the present study, low levels of P-CTX-1 were detected in a lobster (Panulirus penicillatus) and an octopus (Octopodidae spp.). This confirmed their important roles in the transfer of CTXs through marine food web. Although pufferfish and porcupinefish have been defined as key vectors to cause puffer fish poisoning, they may also induce CFP as P-CTXs were present in an omnivorous pufferfish (Arothron nigropunctatus) and two carnivorous porcupinefishes (Diodon hystrix and Diodon liturosus) collected from the ciguatoxic areas at
concentrations above the threshold levels for the prevention of acute CFP induction. Therefore, the control of CFP should also focus on invertebrates, pufferfish and porcupinefish. Instead of MNA, the confirmation of the presence of biotoxins in meal remnants using LC-MS/MS may be crucial to prevent misdiagnosis of various seafood toxin-related illnesses.

In the current study, P-CTX-1 was isolated and purified from viscera of moray eels as authentic standard to develop two analytical methods for ciguatoxic fish screening. The employment of ASE coupled with LC-MS/MS provided a rapid, reliable and direct measurement of P-CTX levels in consumable fish muscle. The use of fish blood, on the other hand, allows for a less-destructive means of identifying and quantifying P-CTXs. This method can be more reliable as matrix effects in fish blood were less than those in fish muscle. Total ciguatoxicity in fish blood can also be used to estimate total ciguatoxicity in consumable fish muscle as significant positive relationships were found between P-CTX-1 level and total ciguatoxicity in these matrices. Further studies on P-CTX pharmacokinetics in coral reef fishes may provide additional information to reinforce the use of fish blood in ciguatoxic fish screening. Surgeonfish and parrotfish were identified as the key vectors to link CTX producers to secondary consumers in the present study. They fed selectively on biofilm as major food source, and therefore biofilm could be an important source of P-CTXs in the ciguatoxic sites in the Republic of Kiribati. The presence of P-CTX-1 in herbivorous fishes demonstrated that biotransformation of GTXs, P-
CTX-2 and P-CTX-3 to P-CTX-1 occurred once CTX-carrying agents were consumed by herbivorous fishes. In addition to coral reef fishes, invertebrates can also transfer P-CTX in the food web and contribute to ciguatoxicity at higher trophic levels. P-CTX-1 was found to have a TMF > 1, indicating P-CTX-1 was biomagnified within the ciguateric marine food web.

Biofilm is an aggregation of bacteria, algae, fungi and detritus that form a complex micro-environment on the hard surface. As there have been growing evidence showing that benthic cyanobacteria could be CTX-producers and biofilm was found to be an important food source to grazers that linked CTX-producers to secondary consumers, it is important to clarify the roles of cyanobacteria and dinoflagellates in the CTX production. Because of a lack of P-CTX authentic standards, only P-CTX-1, P-CTX-2 and P-CTX-3 were quantified in invertebrates and fishes collected from the ciguatoxic and the reference sites in the Republic of Kiribati. It is important to identify other CTX precursors so as to provide a clear picture on the biotransformation of these toxins in the marine food web. Studying the trophodynamics of CTXs in CFP-endemic regions of the Pacific and Indian Oceans and Caribbean Sea may also help to characterize the sources and fates of these toxins in the marine environment.
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