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Comparative Analysis of Purified Pacific and Caribbean Ciguatoxin Congeners and Related Marine Toxins Using a Modified ELISA Technique

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The monoclonal antibody to ciguatoxin (CTX) produced from a hybridoma cell line was assayed for the detection of four congeners of CTX: Pacific ciguatoxin-1 (P-CTX-1), Pacific ciguatoxin-2 (P-CTX-2), Pacific ciguatoxin-3 (P-CTX-3), and Caribbean ciguatoxin-1 (C-CTX-1) and related marine toxins, including domoic acid, palytoxin, and okadaic acid, using a modified enzyme-linked immunosorbent assay (ELISA). Lower detection limits were assessed and linearity was statistically established ($P<0.05$) for P-CTX-1, P-CTX-2, and P-CTX-3 and C-CTX-1 at concentrations ranging from 0 to 5.00 ng, while the other marine toxins showed statistically insignificant cross-reactivities at similar concentrations. Thus, the monoclonal antibody to CTX is able to specifically detect various CTX congeners at levels comparable to those naturally occurring in ciguatoxic fish. J. Clin. Lab. Anal. 20:121–125, 2006.

Key words: ciguatoxin; Pacific ciguatoxin; Caribbean ciguatoxin; ciguatera; monoclonal antibody

INTRODUCTION

Ciguatera seafood poisoning, affecting more than 50,000 people annually, is caused by the ingestion of contaminated reef fishes that have accumulated ciguatoxins (CTXs) in their tissues (1). Produced by the marine dinoflagellate *Gambierdiscus toxicus*, CTXs are stored in the tissues of both herbivorous and carnivorous reef fishes and can be passed through the food chain, ultimately causing gastrointestinal, neurological, and cardiovascular disorders in humans (2).

Slight variations in CTXs can be characterized by the geographical regions in which they are commonly found; these include Pacific ciguatoxin, denoted by P-CTX, Caribbean ciguatoxin, C-CTX, and Indian Ocean ciguatoxin, I-CTX. Both P-CTX and C-CTX have unique molecular chains of 13 and 14 joined ether rings ($C_{62}H_{92}O_{19}$), respectively, as their basic structure. Nine of these transfused rings form a ladder that is similar in all CTXs, with structural modifications on different congeners seen mainly in the termini of the toxin (3). The main CTXs in the Pacific, present in different relative amounts in fish, are P-CTX-1, P-CTX-2, and P-CTX-3, with more than 20 additional congeners having been identified in recent years (4,5). Caribbean CTXs differ from Pacific CTXs and are generally found to be lower in both toxicity and polarity. Other toxins, such as maitotoxins and scaritoxins can also occur in ciguatoxic fish extracts, though their potential roles in clinical poisoning are unclear. Maitotoxins, produced by *Gambierdiscus toxicus*, are toxic via the intraperitoneal route, though they are 100 times less toxic orally, while CTX has been shown to be equipotent (6).

Marine toxins such as domoic acid, palytoxin, and okadaic acid have also been implicated in mammalian disease outbreaks and often utilize molecular mechanisms of action similar to that of CTXs, primarily affecting cellular ion balance and regulation.

Lipid-soluble CTX induces membrane depolarization by activating sodium channels, leading to increased permeability to sodium ions in nerve cells. Domoic acid,

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a neurotransmitter amino acid produced by the marine diatom *Pseudo-nitzschia*, causes amnesiac shellfish poisoning and acts by binding to synaptic receptors of glutamic acid, causing ion channel permeability and subsequent cell death or dysfunction (7). Palytoxin, one of the most deadly marine natural products, is produced by the marine dinoflagellate *Ostreopsis* sp. (8), and found in the marine zoanthid *Palythoa* as well as invertebrates and fish species that live in and around the zoanthid (9). Targeting the Na+/K+–adenosine triphosphatase (ATPase) pump, palytoxin converts the pump into a nonspecific ion channel, thereby short-circuiting membrane function of the cell and eventually causing cell lysis (10). Okadaic acid, a lipophilic phycotoxin produced by the marine dinoflagellate *Prorocentrum* sp. (11), accumulates in mussels and can cause diarrhetic shellfish poisoning. Okadaic acid inhibits serine/threonine protein phosphatases, leading to hyperphosphorylation of sodium ion channels and other proteins responsible for maintaining proper ion balance and neurotransmission (12).

Current methods for the detection of CTX extracted from fish tissue include the mouse bioassay, neuroblastoma assay, and various immunological assays. Hokama (13) developed a simple, rapid immunoassay using a mouse monoclonal antibody to CTX (MAb-CTX) coated with colored latex beads. The test, now marketed as Cigua-Check™ (ToxiTec Inc., Honolulu, HI), has been shown to detect CTX at low levels in clinically implicated crude fish extracts. This test, however, has been questionable at detecting purified CTXs at clinically significant levels, thus the purpose of developing the modified enzyme-linked immunosorbent assay (ELISA) was to determine and compare the MAb-CTX's affinity for different congeners of purified CTXs at varying concentrations, as well as to assay antibody cross-reactivity for selected marine toxins in an effort to verify the commercial version of this immunological method.

MATERIALS AND METHODS

Marine Toxins

Purified Pacific CTXs (P-CTX-1, P-CTX-2, and P-CTX-3) and Caribbean ciguatoxin (C-CTX-1) were purchased from Richard Lewis, University of Queensland, Australia. Purified palytoxin was purchased from Hawaii Biotech (Honolulu, HI), and pure domoic acid and okadaic acid were purchased from Sigma Chemical (St. Louis, MO). Crude extract was obtained from fish clinically implicated in ciguatera outbreaks as defined by the Hawaii Department of Health. Fish tissue was extracted according to Kimura et al. (14) and extract was tested using the mouse bioassay to confirm toxicity. All toxins were stored at –80°C until use and were serially diluted using methanol for experimentation.

Monoclonal Antibody Conjugation and ELISA Development

A MAb-CTX from hybridoma culture was purified by salt fractionation and conjugated to horseradish peroxidase according to Voller and Bidwell (15) using the glutaraldehyde method. The modified ELISA procedure was adapted from Hokama et al.'s (16) protocol. In short, the sharp end of bamboo sticks were dipped in Pentel correction fluid (Pentel of America, Ltd., Torrance, CA) and allowed to air dry overnight. Sticks were dipped in varying concentrations of purified toxins dissolved in methanol, or methanol alone for 30 sec, removed, and air-dried for 5 min. Sticks were subsequently dipped in a 1:500 MAb-CTX horseradish peroxidase conjugate (MAb-CTX-HRP) in Tris buffer with 1% nonfat dried milk to block nonspecific binding for 30 sec, followed by three thorough washes in Tris buffer. Sticks were placed in o-phenylenediamine dihydrochloride (OPD) substrate for 15 min. The reaction was stopped by addition of an equal volume of 1 M sulfuric acid. The resulting color was read using a Bio-Tek Instruments microplate reader (Bio-Tek Instruments, Winooski, VT) at an absorbance of 490 nm. Five replicates of each sample concentration were assayed.

Data Analysis

Sticks were treated using purified toxin dissolved in methanol at 5.00, 1.25, 0.625, 0.156, and 0.078 ng/mL, and methanol alone to serve as a control. The average of the methanol blanks was subtracted from the value of each individual data result ($n = 5$ sticks tested per concentration) for the various concentrations of toxin. Outliers were determined using the Grubbs test and, if present, were eliminated from the data sets. These subtracted results were then averaged to obtain the data points used in linear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA), with the methanol blank being scored as the value "0.00".

RESULTS

After optimizing the experimental conditions and obtaining linear curves for data points using ciguatera-implicated positive fish extract in correlative milligram per milliliter ranges (Fig. 1), pure P-CTX-1 was assayed in the 20–40 ng/mL range to verify the procedure using purified product (data not shown). Because the MAb-CTX proved effective in detecting P-CTX-1 at these levels, the concentration of toxin was decreased in an effort to determine the lower limits and sensitivity of

MAb-CTX using the MAb-CTX-HRP conjugate. This data confirms previous AOAC collaborative studies (17) showing that the MAb-CTX used in the Cigua-CheckTM membrane immunobead assay can detect as little as 0.080 ng/mL of CTX in crude fish extract standard. The MAb-CTX-HRP conjugate was shown to consistently detect purified P-CTX-1, P-CTX-2, and P-CTX-3 at levels ranging from 5.00 to 0.078 ng/mL (Figs. 2 and 3), exhibiting strong R^2 and *P* values (Table 1). C-CTX-1 was also reliably detected in the 1.25–0.078 ng/mL range, however, the optical density values level off slightly at the higher testing limit of 5.00 ng/mL, giving lower R^2 and *P* values, which at the outset make it appear to look statistically insignificant. In fact, C-CTX-1 and P-CTX-3 (Fig. 3) showed the highest optical density readings of all of the purified toxins, making it likely that these congeners have properties that allow more efficient binding to the substrate. Low, nonspecific affinity of MAb-CTX for okadaic acid, palytoxin and domoic acid is shown in

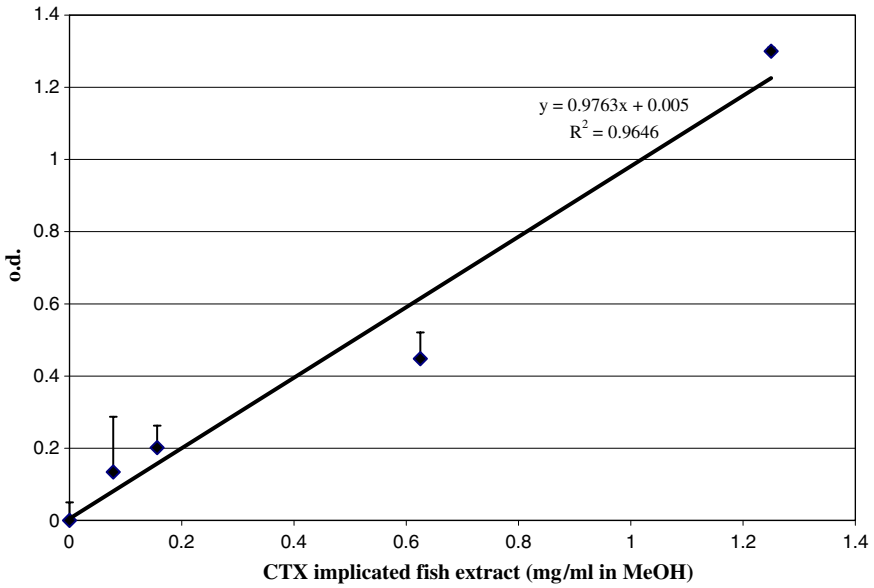

Fig. 1. Results from modified ELISA showing specificity of MAb-CTX for CTX-implicated positive fish extract.

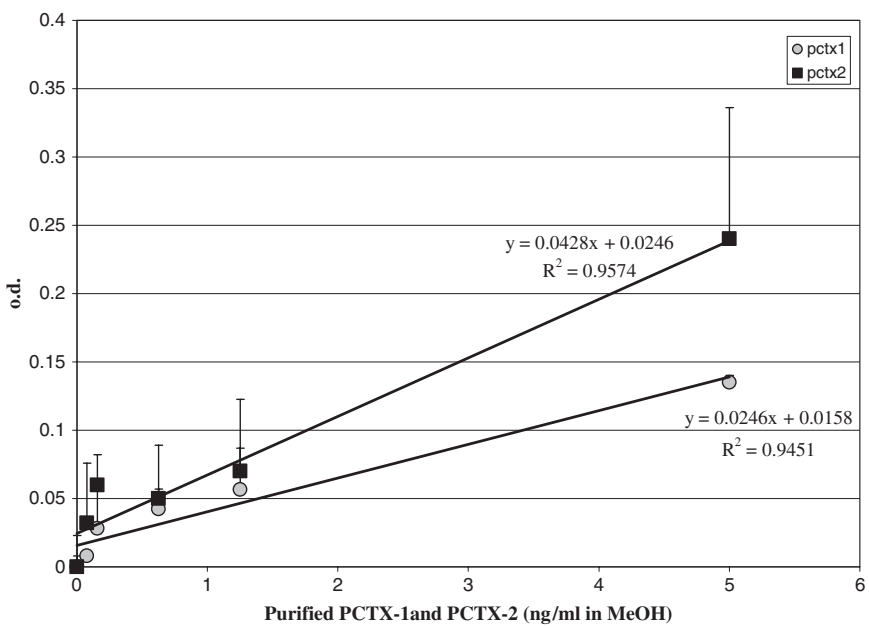

Fig. 2. Results from modified ELISA showing specificity of MAb-CTX for purified P-CTX-1 and P-CTX-2.

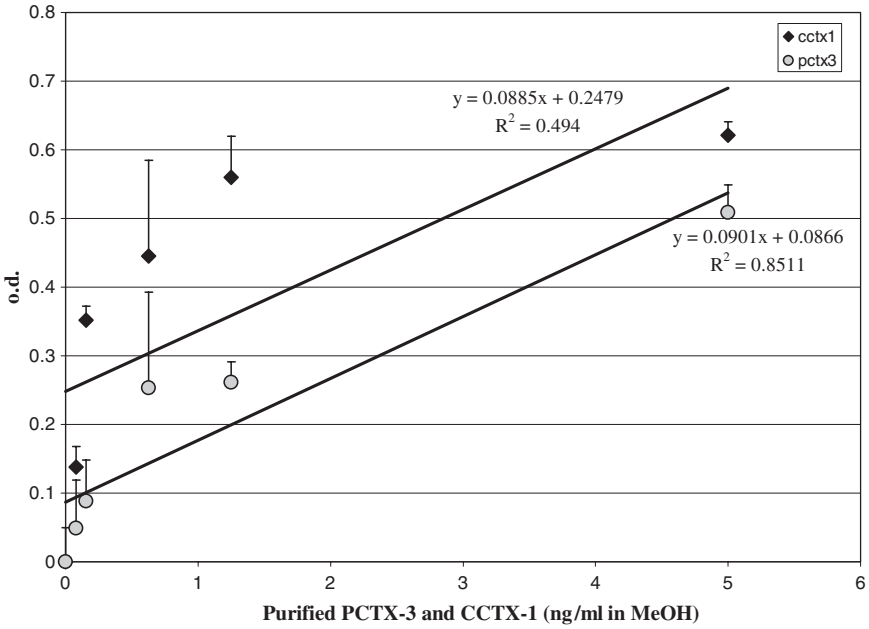

Fig. 3. Results from modified ELISA showing specificity of MAb-CTX for purified P-CTX-3 and C-CTX-1.

TABLE 1. Linear Regression Analyses of MAb-CTX vs. Various Compounds

	Linear regression	
Compound	R^2	*P* value
Ciguatera-implicated extract	0.9646	0.0029
P-CTX-1	0.9451	0.0012
P-CTX-2	0.9574	0.0007
P-CTX-3	0.8511	0.0088
C-CTX-1	0.4940	0.1348
Palytoxin	0.1042	0.5327
Okadaic acid	0.0188	0.7956
Domoic acid	0.3652	0.2039

Fig. 4 with statistically insignificant R^2 and *P* values being demonstrated. It is interesting to note that in other studies performed, okadaic acid appeared to be detected using this method at toxin concentrations higher than 25 ng/mL, suggesting that concentration plays some role in the reactivity and specificity of the MAb-CTX (12).

DISCUSSION

The only commercially available ciguatera detection kit, Cigua-Check, employs a polyvinyl difluoride (PVDF) membrane-based substrate to successfully allow for crude toxin binding following a methanol extraction from fish flesh. The assay, however, has not been able to repeatedly detect purified CTXs in methanol. This is likely due in large part to the matrix of lipids that exist in conjunction with crude methanol fish extracts, allowing for more efficient binding of the small, nonantigenic CTX molecule to the membrane substrate. Purified toxins lack this diverse matrix essential for adequate membrane adherence and detection using this method. We adapted the original immunoassay developed by Hokama (13), which effectively utilizes bamboo sticks coated in Pentel correction fluid, the porosity and composition of which seems to better bind both crude extract and purified toxins, to verify that the MAb-CTX used in testing crude fish can adequately detect purified CTXs, thus providing the rationale for protocol modification.

Efforts were made to minimize error, however, there does exist some variation and nonspecific binding in the optical density readings for different toxins, which can be accounted for in large part by visually undetectable differences in bamboo stick diameter and coating of substrate on the stick, and possible differences in the estimated actual purity of "purified" compounds purchased or received through third-party sources. The modified ELISA assay as described is not suitable for commercial purposes, given that the above factors largely contribute to variations in perceived selectivity and specificity; however, it does serve as a rapid, reliable means for verifying and comparing cross-reactivity with the immunological methodology used in the marketplace for detecting CTX in methanol extracts of fish flesh.

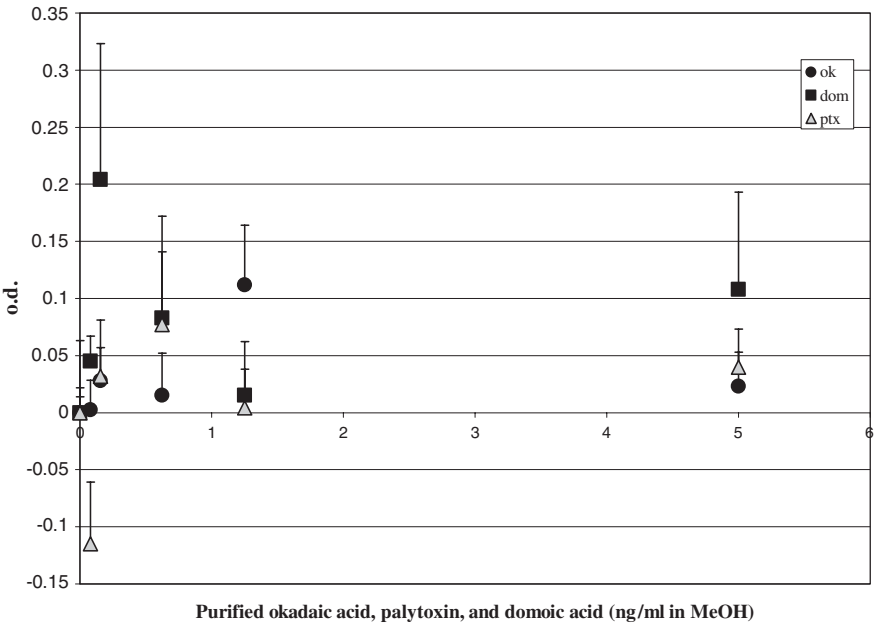

Fig. 4. Results from modified ELISA showing specificity of MAb-CTX for purified okadaic acid (OA), palytoxin (PTX), and domoic acid (DA).

In conclusion, the MAb-CTX-HRP detected both Caribbean and Pacific congeners of CTX with statistical degrees of reliability between 0.078 and 5.00 ng/mL, while other ionophoretic marine toxins showed statistically insignificant cross-reactivity levels with the MAb-CTX at similar concentrations. Moreover, the MAb-CTX-HRP detected P-CTXs and C-CTX-1 at levels comparable to those presumably found in ciguatoxic fish. Further studies to be completed include assaying additional marine toxins such as maitotoxin and brevetoxin using a similar methodology, similar experiments with a chicken polyclonal antibody made to the ABCD fragment of the CTX molecule, as well as following up with cellular methods such as neuroblastoma assays to more thoroughly assess the antibody-antigen reactivity of MAb-CTX to CTXs and related polyethers.

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