CHARACTERIZATION AND IN VITRO CYTOTOXICITY OF TETRODOTOXIN FROM VIBRION ALGINOLYTICUS

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ABSTRACT

Tetrodotoxin (TTX) obtained from the halophilic marine bacterium *Vibrio alginolyticus* was identified by Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC). Among the isolates obtained, 90% of them showed good growth in 10% NaCl and no growth in 0% NaCl. The cytotoxicity of TTX on Vero cells was observed as aggregation, shrinkage, cell rounding and detachment followed by cell death. The *in vitro* non-toxic dilution of TTX (1:32;156.25 ng/mL) determined using MTT assay might find potential therapeutic applications in near future.

Key words: HPLC; HPTLC; MTT; Tetrodotoxin; Vero cells; *Vibrio alginolyticus*

1. INTRODUCTION

Tetrodotoxin, called TTX (Octahydro-12-(hydroxymethyl)-2-imino-5, 9:7, 10a-dimethano-10aH-[1,3] dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol) is a non-protein neurotoxin (Auyoung, 1999). It is a heat stable, white crystalline solid which darkens above 220 °C without decomposition. TTX is not restricted to puffer fish and is widely distributed among various kinds of animals such as the California newt (*Taricha torosa*, Yasumoto et al., 1989), the gastropod mollusks, the blue-ringed octopus (*Hapalochlaena*) and starfishes (Lee et al., 2000). The number of bacterial strains reported to produce the toxin has been increasing and most of the strains belong to the genus *Vibrio*. For instance *Vibrio alginolyticus* isolated from the puffer fish *Fugu vermicularis vermicularis* produced TTX and anhydro-TTX (anh-TTX) (Lee et al., 2000). The probable mechanism of intoxification of TTX in animals has recently been discovered. It is an irreversible sodium ion channel blocker (Do et al., 1990 and Geffeney & Ruben, 2006), which effectively shuts down transmission of nerve impulses leading to paralysis and death in humans (Narahashi, 2001). Even though TTX is a deadly toxin, it also has some therapeutic effects. It prevents ischemic damage of brain that follows stroke (Narahashi, 2001); suppresses chronic pain (Younglai, 2003); relieves the symptoms of withdrawal in opiate addicts and also used as prolonged local anesthetic (Padera et al., 2006).

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In the present study, TTX obtained from *Vibrio alginolyticus* was identified by TLC, HPTLC, HPLC and the non-toxic dilution of the toxin was determined using *in vitro* model.

2. MATERIALS AND METHODS

Screening of *Vibrio alginolyticus*

V. alginolyticus isolates were obtained from Central Institute of Brackish water Aquaculture (CIBA), Chennai, India. The isolates were plated on Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) plate and incubated at 37 °C for 24 – 48 h. The isolates were identified by microscopic examination and standard biochemical tests such as catalase, oxidase, urease, indole, methyl red, Voges-Proskauer, citrate utilization, arginine dihydrolase, lysine decarboxylase and growth on 10 % Sodium chloride (NaCl). *V. alginolyticus* was cultured in beef extract broth (0.5 g glucose , 0.5 g proteose peptone, 0.5 g beef extract, 3.0 g NaCl, 100 mL distilled H_2O, pH - 8.0) at 37 °C for 48 h in a orbital shaker. The culture broth was processed to enrich the toxin content.

Processing of broth

The culture broth was processed according to the procedure of Maoqing & Shum (2003a). The culture broth was centrifuged at 8000 rpm for 10 min at room temperature. Glacial acetic acid equal to 0.1 % by volume of supernatant was added and stirred for 1 h at room temperature. The mixture was heated at 70 – 95 °C for 20 – 30 min and centrifuged to remove scleroproteins. The modification in procedure of Maoqing & Shum (2003a) includes the heating of processed broth at 170 °C for 1 hr to obtain crystals.

The weight of the crystals was determined and the crystals were dissolved in methanol: water (1:1) solvent system. This processed sample was used for the analytical identification of TTX.

Analysis of the processed broth

Spectrophotometric analysis of the processed sample was performed by scanning the sample in the wavelength range of 190 – 1100 nm using UV spectrophotometer (Techcomp 8500, China) and methanol: water (1:1) solvent system as blank. Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} (20 cm x 20 cm). About 20 µL of the sample was spotted on the TLC plate and the chromatogram was developed using the butanol: acetic acid: water (2:1:1) solvent system. The developed plates were sprayed with 10% KOH and baked at 110 °C for 10 min. TTX was observed as blue colored fluorescent spot when viewed under UV lamp of 365 nm and its Rf value was calculated. The developed plate was scanned using TLC scanner (Camag Fenner II, USA). The HPLC analysis of the processed sample was performed on octadecylsilane (ODS) reversed phase adsorption column matrix (250 X 100 mm) using aqueous solution of sodium heptane sulfonate (pH 5.3) as mobile phase using Hitachi D-7000 HPLC system (Hitachi, Ltd., Japan). The column temperature was maintained at 30 °C. The pump pressure was maintained at 3000 psi with the flow rate of mobile phase adjusted to 10 mL/min. The processed sample injected into the column was analyzed at 201 nm for the presence of toxin following Maoqing & Shum (2003b).

Cytotoxicity assay

The Vero cells (African green monkey kidney cells) obtained from King Institute of Preventive Medicine, Chennai, India was used for the cytotoxicity studies. The cells were grown in 24 well plate in Eagle's minimum essential medium (HiMedia) supplemented with 10% fetal bovine serum (Gibco Laboratories) and antibiotics (streptomycin, penicillin, kanamycin, amphotericin B). The cell suspension (10^5 cells/mL) was seeded in every well and incubated at 37 °C for the formation of confluent monolayer in the presence of 5% CO_2 for 48 h. The crude TTX was made to a final concentration of 10 µg/mL. The monolayer of cells was incubated with various dilutions of TTX sample for 24 h. The cell viability was measured using MTT assay using MTT (5 mg/mL) and DMSO as described by Mosmann (1983). Controls were maintained throughout the experiment (untreated wells as cell control and diluent treated wells as diluent control). The assay was performed in duplicates for each dilution of TTX. The mean of the values was used to calculate the cell viability data. A graph was plotted against the % cell viability Vs dilution of TTX.

3. RESULTS

The isolates showed yellow colored round, smooth, highly motile, gram negative colonies. The strain was tested positive for catalase, oxidase, Voges – Proskauer, lysine decarboxylase and negative for urease, indole, methyl red, citrate utilization and arginine dihydrolase. Among the isolates, 90% of them showed good growth in 10% NaCl and no growth in 0% NaCl.

The yield of the crude TTX crystals was 28 mg/mL of the culture broth. In spectrophotometric analysis, two peaks were obtained at 198 nm & 291 nm respectively (Fig. 1). In TLC, the blue colored fluorescent spots were seen when viewed under UV lamp of 365 nm. Its Rf value was calculated as 0.54. The HPTLC chromatogram showed 3 peaks whose Rf values were 0.06 0.57 and 0.84 respectively. The Rf value of 0.57 in the developed chromatogram corresponds to TTX (Fig. 2). The HPLC chromatogram of the sample showed a peak at the retention time of 13.14min (Fig. 3).

The microscopic observation of the Vero cells treated with crude TTX sample showed aggregation, shrinkage, cell rounding and detachment followed by cell death. The percentage viability of the cells was calculated and the graph was plotted (Fig. 4). The non-toxic dilution of TTX sample was determined as 1:32 (156.25 ng/mL) using MTT assay. This finding would serve as a platform for further *in vivo* studies.

4. DISCUSSION

Vibrio alginolyticus could be selectively screened by plating the Vibrio isolates on growth medium supplemented with 10% NaCl (De Poala Jr & Kaysner, 2004). The yield of crude TTX crystals from *V. alginolyticus* culture broth (28 mg/mL) was higher compared to that obtained by Maoqing & Shum (2003a). Their process yielded was 4-6 g of TTX crystals per 100 kg of puffer fish ovaries. The improved culture broth of *V. alginolyticus* could be used for the

mass production of TTX. The pyridine: ethyl acetate: acetic acid: water (15:5:3:4) solvent system showed false positive results in TLC analysis of the processed sample as pyridine showed traces of fluorescence at 365 nm. The chromatographic characterization of the processed sample showed the presence of TTX when compared with the findings of Maoqing & Shum (2003a, b). Since TTX is a heat stable alkaloid and it showed activity on Vero Cells even after processing at 170 ºC where most of the extracellular biologically active molecules of *V. alginolyticus* are denatured.

TTX is being used as local anesthetic, to treat ischemic neuronal injury (IC_{50} = 63 nmol/L, Lysko etal., 1994), to produce prolonged nerve block and in studies on effects of particular receptor in individual cells. Since, TTX binds to sodium ion channels and prevents excessive neuronal depolarization (ionic imbalance), intracellular calcium increase and glutamate release (Lysko et al., 1994). Recently, the usage of TTX has been reported to produce minimal myotoxicity and neurotoxicity in rats (Padera et al., 2006). The toxicity effects of TTX could be wholly reduced by using the non-toxic dilution (1:32; 156.25 ng/mL) of TTX sample determined in our study. The present findings need to be duly evaluated using *in vivo* models before being used in clinical applications.

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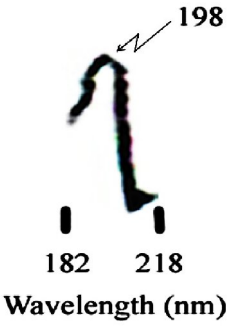

Fig. 1 The UV-spectrophotometric analysis of the processed sample within the wavelength 190 – 1100 nm showed the presence of TTX (λ_{max} - 198nm) in the processed sample.

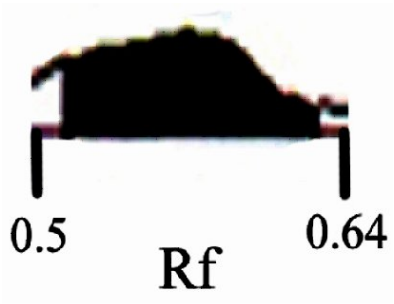

Fig. 2 Showed the presence of TTX (Rf – 0.57) in the HPTLC chromatogram of the processed sample obtained using Camag Fenner II TLC scanner at 366 nm.

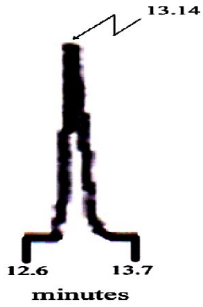

Fig. 3 Showed the presence of TTX (Rt – 13.14 minutes) in the HPLC chromatogram of the processed sample analyzed at 201 nm using D-7000 HPLC system

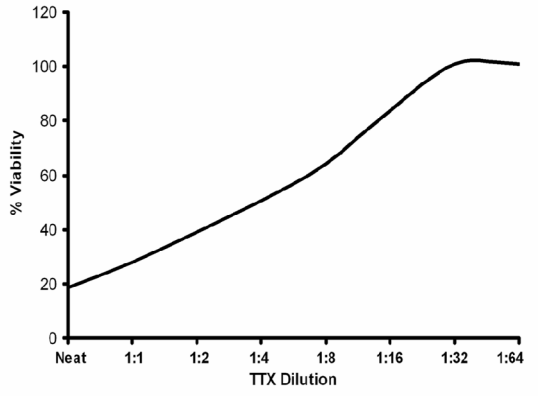

Fig. 4 MTT assay of the TTX sample treated Vero cells after 24 hour showed 100% cell viability at 1:32 dilution as compared with cell & diluent control.

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