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Screening for Tetrodotoxin (TTX) in the Trochid Marine Snail *Umbonium costatum* from the West and South Coasts of Korea Using TTX-specific Monoclonal Antibody in Competitive Enzyme-Linked Immunosorbent Assay (cELISA)

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ABSTRACT

Tetrodotoxin (TTX) is a potent neurotoxin commonly found in pufferfish and various marine invertebrates. Previous studies conducted in southern Taiwan and Japan have reported that species within the genus *Umbonium*, a group of marine trochid snails, act as vectors for TTX. Although these trochid snails are also commonly found on sandy or muddy tidal flats along the west and south coasts of Korea, no studies have yet investigated the presence of TTX in these snails. In this study, we screened *U. costatum*, a species widely distributed along Korea's west and south coasts, for the presence of TTX. In 2023, we collected *U. costatum* specimens from Myeongsa Beach on the south coast and Seonyudo Beach on the west coast, and analyzed their tissues for TTX using a competitive enzyme-linked immunosorbent assay (cELISA). The cELISA results indicated that TTX concentrations in the tissues of all collected snails were below the detection limit. Based on these findings, it appears that *U. costatum* from the west and south coasts of Korea may lack the capacity to accumulate TTX, or the absence of TTX may be due to the lack of TTX-containing prey in their environment. However, further investigations, including monthly toxicity screenings and studies on the TTX tolerance capacity of *U. costatum*, are needed to understand TTX dynamics in this species better.

Keywords: *Umbonium costatum*, Tetrodotoxin (TTX), competitive ELISA, South Korea

INTRODUCTION

Tetrodotoxin (TTX), an extremely potent natural neurotoxin first identified in pufferfish, interferes with the function of voltage-gated sodium channels on nerve cell membranes (Narahashi, 2008; Saoudi *et al.*, 2010). The disruption of nerve signal conduction caused by TTX leads to symptoms such as numbness, dizziness, vomiting, hypertension, and blurred vision. In severe cases, it can result in hypoxia and severe respiratory failure, which can be life-threatening (Noguchi *et al.*, 2011; Cheung and Chan, 2023). Furthermore, due to its thermally stable structure, TTX is resistant to inactivation by cooking (Saoudi *et al.*, 2007), and given the absence of a known antidote for this toxin, it requires considerable caution (Bane *et al.*, 2014). Beyond the well-known presence of TTX in several pufferfish, it has also been confirmed within multiple taxa of marine invertebrates, such as mud snail (Tsujimura *et al.*, 2017; Dao *et al.*, 2020),

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trumpet shell (Costa *et al.,* 2021; Lage *et al.*, 2023), blue-lined or ringed octopus (Williams and Caldwell, 2009; Yamate *et al.*, 2021; Kim *et al.*, 2023), and xanthid crab (Saito *et al.*, 2006; Tsai *et al.*, 2006). marine snails distributed in Portugal (Silva *et al.*, 2012). To date, TTX poisoning resulting from the consumption of toxified gastropods has been confined to subtropical and tropical regions (Yang *et al.*, 1995; Hwang *et al.*, 2002; Liu *et al.*, 2004; Jen *et al.*, 2007; Ha *et al.*, 2023). However, recent studies have reported the presence of TTX in marine mollusks occurring in European waters (Turner *et al.*, 2015; Katikou, 2019). The recent emergence of TTX-toxified marine mollusks in European waters is presumed to be associated with the sea surface temperature increase driven by global warming and the subsequent expansion of TTX-bearing organisms to the further northern area (Otero and Silva, 2022).

Cases of seafood poisoning associated with consuming TTX-bearing marine organisms have been reported globally, with incidents related to the consumption of gastropods being the second most frequent, following those associated with the ingestion of pufferfish (Guardone *et al.* (2020). TTX has primarily been identified in carnivorous gastropods and reported across various taxa. These include small gastropods such as several *Nassarius* species (Hwang *et al.*, 1992; Liu *et al.*, 2004; Luo *et al.*, 2012; Tatsuno *et al.*, 2023) and *Tanea lineata* (= *Natica lineata*) (Hwang *et al.*, 1990); medium-sized gastropods like *N. glans* (Hwang *et al.*, 2005; Taniyama *et al.*, 2009; Ha *et al.*, 2023), *Babylonia japonica* (Noguchi *et al.*, 1981), *Oliva hirasei* (Jen *et al.*, 2014; Lin *et al.*, 2014), and *Neverita didyma* (Han *et al.*, 2023); as well as large gastropods such as *Charonia lampas* (Narita *et al.*, 1981; Costa *et al.*, 2021; Lage *et al.*, 2023) and *Tutufa bufo* (= *T. lissostoma*) (Noguchi *et al.*, 1984). Not limited to carnivorous gastropods, trace amounts of TTX have been detected in the herbivorous species *Phorcus lineatus* (= *Monodonta lineata*) and *Steromphala umbilicalis* (= *Gibbula umbilicalis*), the trochid.

Notably, the trochid marine snail *Umbonium suturale* occurring in southern Taiwan is identified as a natural TTX vector, as Lin and Hwang (2001) reported TTX toxicity of *U. suturale* as 77 mouse units per gram (MU/g). In Korean waters, the trochid marine snail *U. costatum* is widely distributed on the sand beaches and sandy mud tidal flats along the west and south coasts of Korea. However, these snails are often recognized as locally consumed by the villagers on the west and south coasts, and the presence of TTX in the snails' tissues has yet to be examined. In this study, *U. costatum*, a candidate TTX vector, was collected from the south and west coasts of South Korea, and we screened TTX in their tissues with the TTX-specific monoclonal antibody in a competitive enzyme-linked immunosorbent assay (cELISA).

MATERIALS AND METHODS

In February and September 2023, *U. costatum* were collected from Myeongsa Beach In Geoje Island on the south coast of Korea. *U. costatum* was also sampled from Seonyudo Beach on the west coast in September 2023 (Fig. 1 and 2). For TTX screening, their soft tissues were separated from the shell and dissected into muscle and visceral mass. The specimens from Seonyudo, which had shell diameters of less than 10 mm, were pooled into ten groups of five each for analysis due to their small size. The separated tissues were then stored at –80°C until TTX screening.

The screening for TTX in the muscle and visceral mass of the button top shell was analyzed using a EuroProxima Tetrodotoxin cELISA kit (R-Biopharm Nederland B.V., Arnhem, Netherlands). This kit has a detection limit of 9.4 ng/g and a detection capability of 20 ng/g, with a mouse monoclonal antibody specific to TTX. To briefly explain the analysis process, the homogenized tissue samples were mixed with 5 mL of sodium acetate buffer (composed of 300 mL of 0.1 M $C_2H_3NaO_2$ and 200 mL of 0.1 M CH_3COOH, at pH 4.8) and centrifuged at 4000 × g. Fifty microliters of the supernatants from the samples and the TTX standards (ranging from 0.6 to 20.0 ng/mL) were added into microplate wells

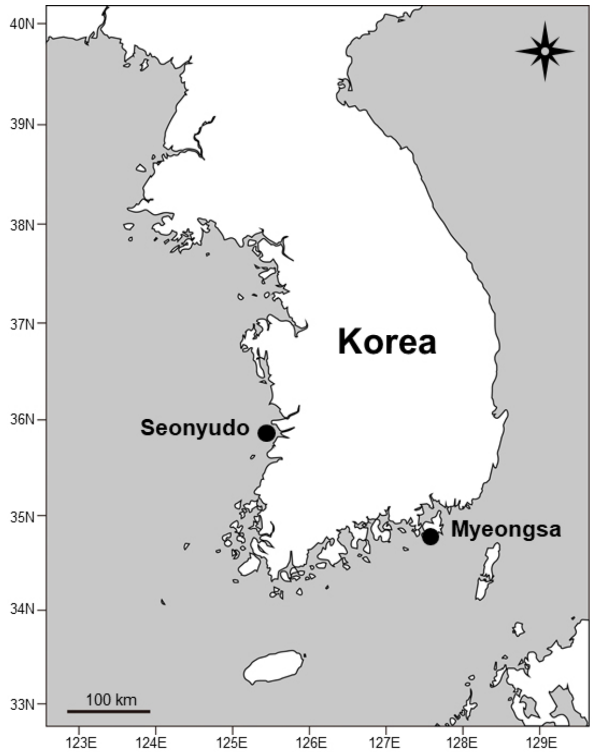

Fig. 1. The sampling sites for *Umbonium costatum* were located in sandy areas along the southern coast (Myeongsa) and the western coast (Seonyudo) of Korea.

pre-coated with TTX. Following the addition of 50 μL of the mouse anti-TTX primary antibody to each well, the samples were incubated in the dark at room temperature for 30 minutes. After washing the wells, 100 μL of secondary antibody (horseradish peroxidase-labeled anti-mouse antibody) was added, and the samples were then incubated for another 30 minutes in the dark at room temperature, followed by another wash. Then, 100 μL of substrate solution (hydrogen peroxide/tetramethylbenzidine) was added to visualize the TTX-antibody complex, followed by a 30-minute incubation in the dark. The reaction was stopped with the addition of sulfuric acid. Using a VersaMax microplate spectrophotometer (Molecular Devices, San Jose, CA, USA), the absorbance of the TTX-antibody complex was measured at 450 nm. TTX concentrations were then determined using RIDASOFT Win software (R-Biopharm, Darmstadt, Germany).

RESULTS AND DISCUSSION

This study presents the first tetrodotoxin (TTX) screening in *U. costatum*, a candidate species for TTX-bearing organisms found in Korean waters. The cELISA showed strong linearity, with a coefficient of determination (r^2) of 0.99 (Fig. 3). The cELISA results revealed that TTX levels were below the detection limit in all tissues of the specimens collected from Myeongsa Beach in March and September, as well as from Seonyudo Beach in September (Table 1). Therefore, *U. costatum* from the west and south coasts of Korea do not appear to be a vector of TTX. However, a more sensitive technique, such as liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), is recommended to confirm these findings.

Lin and Hwang (2001) first reported TTX toxicity in the trochid snail *Umbonium suturale* tissues, which was found in the digestive gland of the starfish

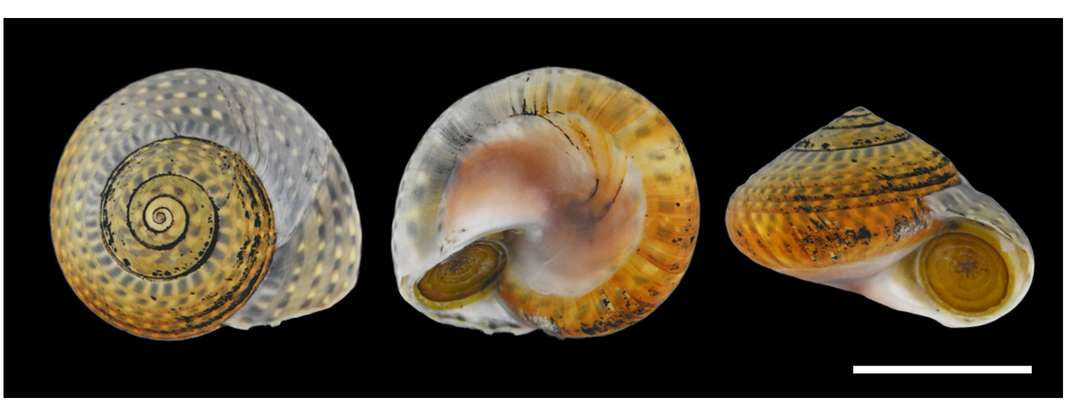

Fig. 2. Photographs of *Umbonium costatum* specimen collected from Myeongsa. Scale bar = 1 cm.

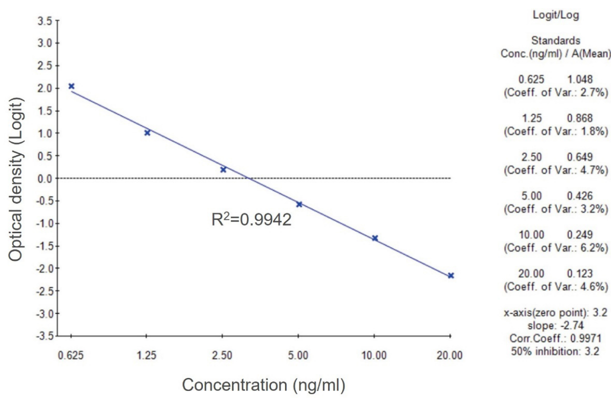

Fig. 3. **The standard curve for competitive ELISA of tetrodotoxin, showing the correlation between tetrodotoxin concentration (ng/ml) and optical density (logit). TTX standards were prepared at 0.625, 1.25, 2.50, 5.00, 10.00, and 20.00 ng/ml.**

Astropecten scoparius in southern Taiwan. The recorded toxicity level was 77 mouse units (MU)/g. One mouse unit is defined as "the amount of toxin required to kill a 20 g ICR (Institute of Cancer Research) strain male mouse within 30 minutes of intraperitoneal injection" (Lin and Hwang, 2001). Remarkably, the highest TTX toxicity in *A. scoparius* tissue reached 16,821 MU/g, though these levels fluctuated seasonally. Lin and Hwang (2001) suggested that *A. scoparius* accumulates TTX by preying on *U. suturale*, as the toxicity in the snails' tissues also showed distinct seasonality.

In contrast to *U. suturale* in southern Taiwan, *U. costatum* from the west and south coasts of Korea were devoid of TTX, despite belonging to the same genus. This difference in toxicity could be partly explained by variations in prey profile due to geographical or seasonal differences. An exogenous pathway is the most likely mechanism for acquiring TTX (Noguchi *et al.*, 2006; Itoi *et al.*, 2018). TTX can be acquired exogenously either through the ingestion of TTX-producing bacteria (Kogure *et al.*, 1988; Do *et al.*, 1991) or by consuming organisms that already contain TTX, leading to its accumulation (Lin and Hwang, 2001; Salvitti *et al.*, 2015; Okabe *et al.*, 2019). Thus, TTX toxicity often depends on diet, which can vary regionally.

The absence of TTX in *U. costatum* from Korean waters may also be linked to species-specific differences in the ability to accumulate TTX within the same genus. This phenomenon has been observed in mud snails (*Nassarius* spp.). According to Luo *et al.* (2012), two species of mud snails, *Nassarius sinarum* (= *N. semiplicatus*) and *N. variciferus*, collected from the same location and period in Lianyungang, China, exhibited a marked difference in TTX toxicity. While *N. sinarum* showed significant TTX toxicity, with a maximum of 846 MU/g, *N. variciferus* exhibited only weak toxicity at 10.6 MU/g, despite both species belonging to the same genus (Luo *et al.*, 2012).

High TTX toxicity is often associated with reproductive cycles. In female *Takifugu niphobles* from Kanagawa, Japan, TTX concentrations increased during gonadal maturation in April and peaked during the spawning period from May to July (Itoi *et al.*, 2016). Similarly, in *A. scoparius* from Taiwan, toxicity was highest during the ripening period in September and November (Lin and Hwang, 2001), suggesting that reproductive maturity may be linked to increased toxicity. However, information on the annual reproductive cycle of *U. costatum* from the west and south coasts of Korea is not yet available, leaving it unclear whether the absence of TTX in the snails' tissues is related to their reproductive cycle.

In conclusion, we screened for TTX toxicity in the trochid snail *U. costatum* from the west and south coasts of Korea using TTX-specific monoclonal

Table 1. The result of TTX screening in the muscle and visceral tissues of *Umbonium costatum* using cELISA

Species	Sampling site	Date	N	TTX μg/g tissue	
				Muscle	Visceral mass
Umbonium costatum	Myeongsa	Feb-2023	10	N.D.	N.D.
		Sep-2023	10	N.D.	N.D.
	Seonyudo	Sep-2023	50	N.D.	N.D.

antibodies in a cELISA. Our results indicated that the TTX levels in 70 specimens were below the detection limits. The absence of TTX in *U. costatum* may be due to species-specific differences in TTX accumulation, although further studies are necessary to confirm this hypothesis.

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