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Research Note

Screening of Tetrodotoxin (TTX) in Red-mouth Frog Shell *Tutufa bufo* (Röding, 1798) from Jeju Island Using Competitive ELISA (cELISA)

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ABSTRACT - Tetrodotoxin (TTX) is a potent neurotoxin found in various marine organisms, including pufferfish and gastropods, which poses significant health risks when consumed improperly. *Tutufa bufo*, a frog shell species in Japan reported to contain TTX, has recently been caught in the southern waters of Jeju Island. However, information on its presence in *T. bufo* distributed in Korean waters is not available. In this study, we screened two specimens of *T. bufo*, a large predatory gastropod, collected from Moseulpo, Jeju Island, Korea, for the presence of TTX using a competitive enzyme-linked immunosorbent assay (cELISA). Soft tissues, including the anterior salivary gland, buccal mass, digestive gland, gills, gonad, kidney, muscle, and posterior salivary gland, were analyzed for TTX levels. The cELISA results showed that the TTX concentrations in all tissues were below the detection limit. However, owing to potential individual, regional, and seasonal variations in TTX accumulation, further studies with additional samples collected during different seasons are necessary to accurately determine the risk of TTX accumulation in *T. bufo* in Korean waters.

Key words: *Tutufa bufo*, Tetrodotoxin, Competitive ELISA, Jeju Island

Tetrodotoxin (TTX) is a potent neurotoxin that was first discovered in pufferfish and has since been detected in various taxa, including gastropods[1,2)], blue-ringed octopuses[3,4)], and xanthid crabs[5,6)]. TTX poisoning often occurs due to the improper consumption of marine organisms containing TTX[7,8)]. The intoxication is accompanied by symptoms of paralysis, dizziness, and vomiting, and in severe cases, can lead to death from respiratory failure, highlighting the need for caution with this toxin[9,10)]. Since TTX poisoning resulting from the consumption of gastropods constitutes a significant proportion following that from pufferfish[11)], information of toxins in potentially edible gastropods is required to prevent TTX poisoning. In 1981 and 1982, TTX was confirmed in *Tutufa bufo* (=*T. lissostoma*) collected from Suruga Bay, Shizuoka Prefecture, Japan, using thin-layer chromatography, gas chromatography-mass spectrometry, and H-NMR spectroscopy, with a maximum toxicity level of 700 mouse units per gram (MU/g) in the digestive gland determined by mouse bioassay[12)]. In Korea, the distribution of *T. bufo* was first confirmed in 2015 in the subtidal area near Bumseom on the southern coast of Jeju Island, Korea[13)]. However, information on TTX in *T. bufo* distributed in Korea is lacking. In this study, TTX screening was performed using a TTX-specific competitive ELISA.

In December 2022, two specimens of *T. bufo* were collected by fishermen in Moseulpo (Fig. 1). These frog shells had shell lengths of 17.4 cm and 22.2 cm, and tissue wet weights of 93.0 g and 128.5 g, respectively. Their external sculpture is characterized by spinous tubercles, and they possess a large, orange-colored aperture (Fig. 2A). For TTX screening, their soft tissues were separated from the shell and dissected into muscle (M), gills (GL), buccal mass (BM), kidney (KD), anterior salivary gland (ASG), and posterior salivary gland (PSG) (Fig. 2B). However, the gonad (GND) and digestive gland (DG) could not be separated through dissection (Fig. 2B); therefore, these two tissues were combined for analysis. The separated tissues were then stored at -80°C until the TTX screening. The total

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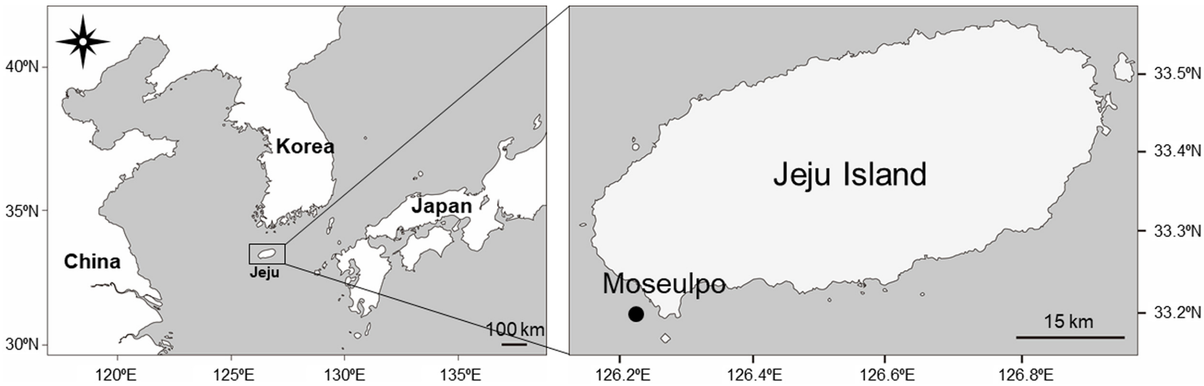

Fig. 1. The sampling sites for the frog shell were located alnog the southern coast of Jeju Island, Korea.

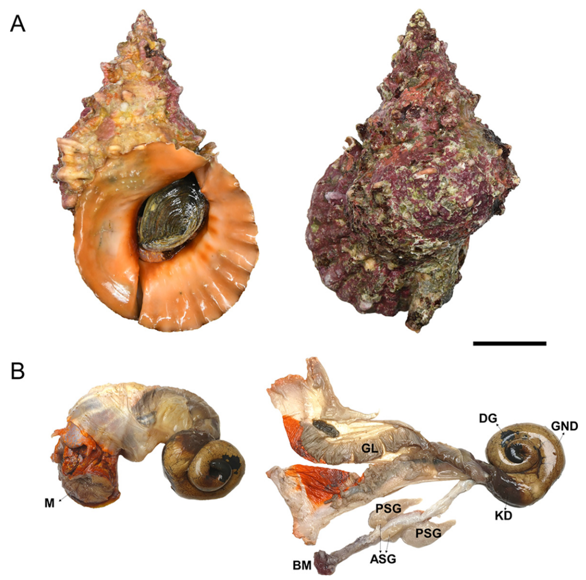

Fig. 2. Photographs of *Tutufa bufo* specimens collected from Moseulpo, Scale bar = 5 cm (A). Dissected anatomical view of *Tutufa bufo* analyzed in this study, showing tissues including the anterior salivary gland (ASG), buccal mass (BM), digestive gland (DG), gills (GL), gonad (GND), kidney (KD), muscle (M), and posterior salivary gland (PSG) (B).

TTX levels in each tissue from *T. bufo* were determined using a EuroProxima Tetrodotoxin cELISA kit (R-Biopharm Nederland B.V., Arnhem, Netherlands). This kit has been validated for TTX screening with decision limits of 9.4 ng/g and a detection capability of 20 ng/g, and can detect concentrations approximately 190 times lower than the safety criterion of 10 MU/g (= 1.78 μg/g)[14] for TTX in seafood, set by Ministry of Food and Drug Safety of the Republic of Korea. According to the manufacturer's protocol for shellfish sample preparation, 5 mL of sodium acetate buffer (comprising 300 mL of 0.1 M $C_2H_3NaO_2$ and 200 mL of 0.1 M CH3COOH, pH 4.8) was mixed with the homogenized tissues. The supernatant obtained by centrifuging the homogenate at 4000×g was added to each well of microplate pre-coated with TTX. The TTX solutions ranging from 0.6 to 20.0 ng/mL were used as standards. After incubating with the TTX-specific primary antibody for 30 minutes in the dark, the wells were washed, followed by incubation with the secondary antibody (Horseradish peroxidase-labeled anti-mouse antibody) for another 30 minutes in the dark. After washing, the substrate solution (hydrogen peroxide/tetramethylbenzidine) was added and

Table 1. TTX screening results for each tissue of *Tutufa bufo* using cELISA

Sample	*Tutufa bufo*1	*Tutufa bufo*2
TTX concentration (ng/g tissue, Average ± SD)		
Muscle	BDL[1)] (4.38±0.12)	BDL (4.27±0.17)
Gills	BDL (8.70±0.05)	BDL (7.88±0.10)
Buccal mass	BDL (4.98±0.15)	BDL (6.39±0.24)
Kidney	BDL (8.60±0.50)	BDL (8.81±0.25)
Anterior salivary gland	BDL (5.92±0.30)	BDL (7.50±0.5)
Posterior salivary gland	BDL (9.10±0.15)	BDL (5.98±0.07)
Gonad and digestive gland	BDL (8.07±0.45)	BDL (6.92±0.61)
TTX μg in individuals	BDL	BDL

[1)]BDL: below the detection limit (<9.4 ng/g).

incubated for 30 minutes, and the reaction was terminated by adding sulfuric acid. TTX concentrations (μg/g tissue) were calculated from optical density measurements at 450 nm using a spectrophotometer (Molecular Devices, San Jose, CA, USA) and a standard curve with RIDASOFT Win software (R-Biopharm, Darmstadt, Germany).

In this study, TTX concentrations were found to be below the detection limit (<9.4 ng/g) in all tissues of two *T. bufo* specimens collected from Moseulpo, Jeju Island (Table 1). The individual and regional variations in TTX content observed in TTX-containing organisms[15], along with the finding that toxic organisms collected from the same site exhibit identical TTX and TTX analogue profiles[16], support the hypothesis that TTX is acquired from dietary sources and accumulates in higher trophic level organisms through the food chain. Therefore, toxification depends on diet, and the values observed in this study may result from the low abundance of TTX-containing prey organisms in Korean waters. Additionally, toxicity can vary depending on the collection period, which may be associated with the reproductive period[17,18]. The *T. bufo* specimens analyzed by Noguchi et al. (1984)[12] were collected in May, September, and October, which do not exactly coincide with the collection period of this study. Thus, differences in toxin levels due to the collection time cannot be excluded. Therefore, additional sample acquisition and analysis are considered essential for accurately determining the toxin levels. Future studies should adopt a broader sampling strategy that encompasses various seasonal periods and multiple locations to gain a comprehensive understanding of the TTX accumulation dynamics in *T. bufo*. Furthermore, investigating the dietary habits of these organisms in relation to TTX content could provide insights into the ecological interactions influencing toxin acquisition within food webs.

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국문요약

본 연구는 제주도 모슬포 해역에서 채집된 대형 포식성 복족류인 붉은입두꺼비고둥(*Tutufa bufo*) 두 개체를 대상으로 테트로도톡신(TTX) 존재 여부를 조사하였다. 일본에서 채집된 붉은입두꺼비고둥의 내장에서 TTX가 검출된 사례가 보고된 바 있으며, 최근 제주도 남부 해역에서 해당 종이 혼획되고 있으나, 한국 해역에 분포하는 붉은입두꺼비고둥의 TTX 축적에 대한 정보는 부족하다. 이에 본 연구에서는 경쟁적 효소면역분석법(cELISA) 을 사용하여 붉은입두꺼비고둥의 주요 연조직(전타액선, 구강부, 소화선, 생식소, 아가미, 신장, 근육, 후타액선)을 분석하였다. 분석 결과, 모든 조직에서 TTX 농도는 검출 한계 미만으로 나타났다. 그러나 TTX 축적에는 개체 간, 지역적, 계절적 변동 가능성이 존재할 수 있으므로, 한국 해역에서 붉은입두꺼비고둥의 TTX 축적 위험을 정확하게 평가하기 위해서는 추가적인 시료 확보와 계절별 연구가 요구된다.

Conflict of interests

The authors declare no potential conflict of interest.

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