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Paralytic toxin profiles of xanthid crab *Atergatis floridus* collected on reefs of Ishigaki Island, Okinawa Prefecture, Japan and Camotes Island, Cebu Province, Philippines

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Abstract: Attempts were made to assess the toxicity and to analyze paralytic toxin profiles of xanthid crab *Atergatis floridus* collected on two reefs on the left- and the right-side, tentatively designated as site A and B, separated by the passage at the outside of Kabira Bay in Ishigaki Island, Okinawa Prefecture, Japan in comparison with those of the same crabs from Camotes Island off the eastern coast of Cebu Island, Cebu Province, Philippines. They were dissected into 4 parts; carapace, viscera, appendage and muscle of appendage. Muscle of appendage was highly toxic, and the maximum toxicity of 4,641±972MU/g as paralytic shellfish poison (PSP) was recorded in the specimens from the right-side reef (site B). Toxicity assays showed that all of them were toxic irrespective of the crab-collecting years, locations, and tissues, and in addition to these, there seemed to be marked narrow regionality and individual variation of toxicity and toxin profiles. Toxicity of Ishigaki specimens was seemed to be higher than that of Camotes specimens. Toxin profiles of the viscera of *A.floridus* were examined by high performance liquid chromatography-fluorescent detection (HPLC-FLD) analysis. In the viscera of *A.floridus* from site A in June, 2007, relative abundances (mole %) of carbamoyl-*N*-hydroxyneosaxitoxin (hyneoSTX), neosaxitoxin (neoSTX), and saxitoxin (STX) were high (98%), and only 2% of gonyautoxin 2(GTX2) were contained in addition to similar amounts (3%) of decarbamoylsaxitoxin (dcSTX). Its viscera from site B in the same month possessed GTX2 (36%) and STX group (63 %) predominantly, and only 1% of GTX1 was contained in addition to similar amount (2%) of STX. Their viscera possessed STX group as the major components (89%) along with the GTX4 (11%) as the minor. On the other hand, PSP compositions of the viscera of Camotes specimen resembled to that of the viscera from the specimens on site A in Kabira Bay with higher GTX4 but lower hyneoSTX. A solitary outstanding difference of toxin profiles in both crabs was the occurrence of tetrodotoxin (TTX) in the Camotes specimen due to the results of HPLC-FLD and gas chromatography-mass spectrometry (GC-MS) analysis.

Keywords: Xanthid Crab, *Atergatis floridus*, Paralytic Shellfish Poison, Tetrodotoxin, Ishigaki Island, Camotes Island, Philippines, HPLC-FID, GC-MS, Gonyautoxin, Saxitoxin

1. Introduction

Paralytic shellfish poison (PSP), a most hazardous marine toxin, mainly originates in toxic marine dinoflagellates species of the genera *Alexandrium*, *Gymnodinium* and *Pyrodinium*, are accumulated in many species of marine organisms such as crabs and filter-feeding organisms such as bivalve mollusks [1, 2, 3]. These organisms can act as potential toxin vectors and pose a threat to human health. Within these PSP containing organisms, it has so far been reported that three species of xanthid crabs, "subesube-manjyugani" *Atergatis floridus*, "umore-ougigani" *Zosimus aeneus*, and "tsubuhiraashi-ougigani" *Platypodia granulosa*, in the family Xanthidae inhabiting tropical and subtropical areas, contain PSP such as saxitoxin (STX) and neosaxitoxin (neoSTX) [4, 5, 6]. One of these toxic crabs, *A. floridus* (Floral egg crab) widely inhabits even in temperate area as well as in tropical and subtropical areas while the other two species not in temperate area. In Japan, it inhabits also the Japan Proper and adjacent islands of the Amami and the Ryukyu but its toxicity and toxin profile varied [7, 8, 9]. There are a number of unclarified points regarding toxification mechanisms of these crabs until now.

The present report therefore aims at presenting basic data on it. Then, at first, we tried to collect *A.floridus* specimens in Ishigaki Is., Okinawa Prefecture, the southern-east area in Japan, and the Camotes Is. in the Camotes Sea off the eastern coast of Cebu Island, Cebu Province, Visayas region of the Philippines. By using these samples, attempts were made to examine two points as follows. The first is to examine details of toxicity and the toxin profiles of *A.floridus* between the two reefs, which are separated by a passage, within very close distance as a part of the study on toxification mechanism of toxic xanthid crabs. In this connection, the second is to examine details of toxicity and the toxin profiles of *A.floridus* from two subtropical areas, Ishigaki Is. and Camotes Is. This paper deals these results obtained.

2. Materials and Methods

2.1. Materials

Figure 1 shows two sampling locations of reefs in Kabira Bay, Ishigaki Island, Okinawa Prefecture, Japan. There are the two reefs, on the left- and the right-side at the outside of the Bay, which are separated by the passage. Here, they are tentatively designated as site A (N24.46934, E124.14392) and B (N24.46630, E124.14685), respectively.

Total fifteen crab specimens were collected on reefs in every June, 2007, 2009 and 2010. At an ebb-tide, these reefs appear and specimens of xanthid crab were collected easily by hand. On the other hand, on reefs along San Francisco (N10.64762, E124.38206) in Camotes Is., Cebu Province, Visayas region of Philippines (Figure.2), five specimens were collected by fishermen using crab cages in August 2009. All the specimens mentioned above were immediately frozen after capture, transported by air to our laboratory, and kept frozen at -20°C prior to identification and toxin profiles analysis. All of them were identified as the xanthid crab *Atergatis floridus* (Linnaeus, 1767) by Dr. M.Shimomura, one of our authors, from the Kitakyushu Museum of Natural History & Human History (Figure.3, [10]).

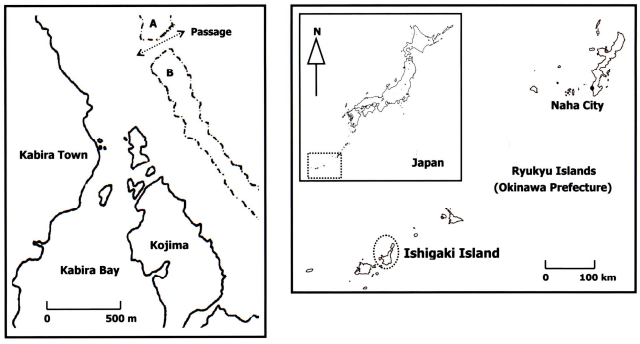

Figure 1. *Map showing crab-collecting locations in Ishigaki Island. The location of Ishigaki Island in Japan is shown in the map to the right. The map on the left shows an enlarged image of Ishigaki Island to pinpoint the sampling location.*

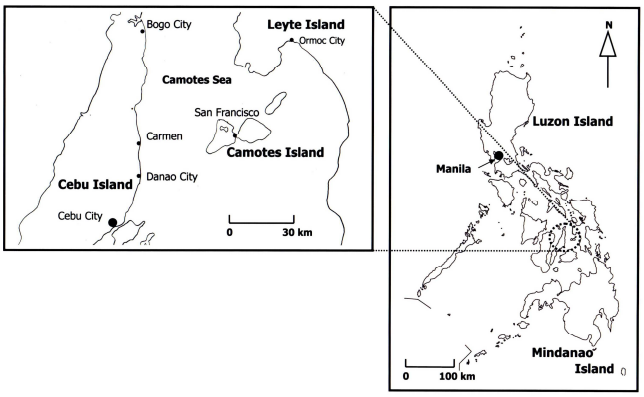

Figure 2. *Map showing crab-collecting locations in Camotes Island. The location of Camotes Island in the Philippines is shown in the map to the right. The map on left shows an enlarged image of Camotes Island to pinpoint the sampling location.*

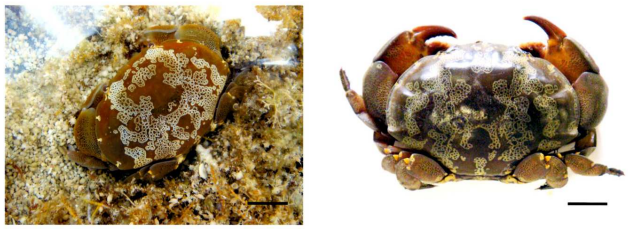

Figure 3. *Toxic xanthid crab Atergatis floridus; (I) A live specimen on reefs outside of Kabira Bay, Ishigaki Island, Okinawa Prefecture, Japan is shown to the left. (II); A specimen on reefs along the town of San Francisco in Camotes Is., Philippines is shown to the right. (Scale bar = 1.0cm)*

2.2. Assay for Lethal Potency

Each crab was partially thawed and dissected into four anatomically different parts: carapace, viscera including hepatopancreas, reproductive organs, and intestines, appendages torn off from each crab specimen and muscles

in the appendages, which called muscles below. We examined toxicity for each tissue by the standard bioassay for PSP [11]. Lethality was expressed in mouse units per gram of crab specimen tissue (MU/g), where one MU is the amount of intraperitonially (*i.p.*) administered toxic material required to kill a 18-20g male mouse of the ddY strain in 15 min.

2.3. Purification of Toxins

Each viscera from crab specimens collected on reefs (site A and B) in Ishigaki Is., June, 2009 were used as material, and then 3 volumes of 1% acetic acid in 80% methanol was added. The mixture was homogenized for 3 min and extracted under reflux. This operation was repeated on the residue twice after filtration. The filtrate was combined and, concentrated under reduced pressure. Extracts (total toxicity: 1,172MU and 249MU as PSP for the left- and the right-side reef crab specimen, respectively) were defatted with dichloromethane, and the aqueous layer was partially purified by successive treatment on activated charcoal (Wako) and Bio-Gel P-2 (Bio-Rad. Lab.) column chromatography. Conditions for each procedure were similar to those in the previous studies [12]. Toxicity was detected exclusively in the 0.03M AcOH fraction obtained from Bio-Gel P-2 column chromatography. This toxic fraction was concentrated to dryness under reduced pressure, and the partially purified toxin obtained was dissolved in a small amount of water, and analyzed for PSP and tetrodotoxin (TTX) by HPLC-FLD as previously described [1, 12, 13, 14]. A LiChroCART RP-18(e) column (φ 4.0 x 250mm, Merck) was used in combination with the two mobile phases. In addition, an alkali-hydrolysate of this toxin was trimethylsilylated and analyzed by gas chromatography-mass spectrometry (GC-MS), as described below.

Standards of PSP and TTX were used in the previous report [14, 15, 16]. The acidified MeOH extract (7,443 MU as PSP) from the viscera of Camotes specimen was prepared by the same methods was subjected the almost the same methods mentioned above.

2.4. Gas Chromatography- Mass Spectrometry

The trimethylsilyl (TMS) derivative of 2 - amino - 6 - hydroxymethyl - 8 - hydroxyquinazoline (C9 base), was derived from purified toxins and authentic TTX by a previously described procedure [17]. Both TMS derivatives were injected to a Varian gas chromatograph (1200 MS/MS) equipped with a mass spectrometer (Varian CP-3800) according to previously described methods [12, 13].

3. Results and Discussions

As shown in Table, all of the crab specimens used in this study were found to be toxic as expected. The frequency of toxic samples was 100% in Ishigaki specimens and their toxicities were widely distributed in various tissues. Though toxicity assays showed that all of them were toxic and induced paralytic symptoms typical of PSP irrespective of crab-collecting locations (site A and B), there are remarkable individual variations in anatomical distribution of toxicity. Distributions of PSP toxicity scores in each tissue in Ishigaki specimens were as follows; carapace from 183 ± 47 to 807 ± 693 MU/g, viscera from 64 ± 41 to 654 ± 137 MU/g, appendages from 88 ± 40 to 1,257 ± 607 MU/g and their muscle from 1,408 ± 404 to 4,641 ± 972 MU/g (Av. ± S.D.), respectively. On the other hand, all of the Camotes Is. specimens also showed high toxicities in each tissue as follows; viscera (105 ± 56 MU/g), appendages (221 ± 189 MU/g) and their muscle (719 ± 349MU/g) (Av. ± S.D.), respectively. It is also reported that muscle of appendages showed highest toxicity (1,100-5,900 MU/g) irrespective of the specimens of *A.floridus* and *Z.aeneus* from Ishigaki Island [18]. Toxicity of Camotes specimens was seemed to be lower in comparison with that of Ishigaki specimen with the highest score of 5,613 MU/g (Site B, June 200).

Table. *Toxicity of xanthid crab Atergatis floridus collected on the reefs of Ishigaki Island, Okinawa Prefecture, Japan and Camotes Island, Cebu Province, Philipines*

(A) Okinawa Prefecture, Japan							
Month, Year of Collection	Place of Collection		Number of Specimens	Body Weight (g) Av. ± SD	Toxicity (MU/g) : Av. ± S.D. Carapace	Viscera	Appendage	Muscle
June, 2007	Ishigaki Island	Site A	3	18 ± 8	NT	64 ± 41	88 ± 40	NT
		Site B	3	30 ± 3	NT	253 ± 203	113 ± 115	NT
June, 2009		Site A	3	20 ± 5	183 ± 47	484± 255	503 ± 179	1,408 ± 404
		Site B	3	23 ± 4	807± 693	532± 200	856 ± 700	2,538 ± 2,077
June, 2010		Site B	3	23 ± 1	332 ± 49	654 ± 137	1,257 ± 607	4,641 ± 972
(B) Cebu Province, the Philippines							
Month and Year of Collection	Place of Collection		Number of Specimens	Body Weight (g) Av. ± SD	Toxicity (MU/g) : Av. ± S.D. Carapace	Viscera	Appendage	Muscle
August, 2009	Camotes Island		5	11 ± 3	NT	106 ± 56	221 ± 189	719 ± 349

NT; not tested, Av; average, S.D.; standard deviation

As the food poisoning due to ingestion of toxic crabs sometimes occurs in the fisher folk of coastal areas in the Philippines, people should be warned of the potential hazard of this crab in order to prevent its intentional or accidental consumption due to by-catch. Though there is no habit to eat crabs from tropical or subtropical area in Japan,

marine crabs form a part of the diet of many Filipinos. While most species are edible, some are unfortunately toxic to man and other mammals. Since the 1960s, at least seven cases of crab intoxication in Negros Oriental and the nearby Camotes Is., six of which were fatal, occurred [19]. Though the most common toxic species in this region is *Demania cultripes*, *Lophozozymus pictor*, *Z. aeneus* and so on[12, 20, 21, 22], *A.floridus* from Camotes Is. is highly toxic, too. So, it is important to supply food hygienic information on this toxic crab *A.floridus* for fishermen and inhabitants there. Since intake of toxin levels above 3,000 MU is assumed to be lethal to the adult human [23], consumption of some of the specimens of *A.floridus* could prove fatal. It is strongly advised that consumption of crabs living on reefs be avoided. In this connection, the results of lethality tests using whole bodies of all 35 *A.floridus* specimens from Fiji, Is. showed individual lethalities ranged widely from 3.4 to 717 MU/g [24].

The toxin partially purified from viscera of *A.floridus* collected on the reef (site B) in Ishigaki Is., June, 2007, showed a clear peak in HPLC-FLD chromatograms corresponding to the retention time of standards of PSP (Figure 4).

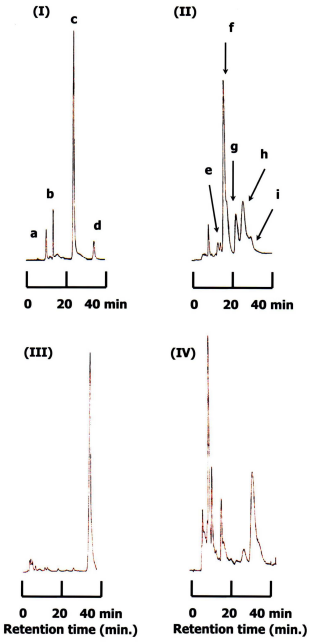

Figure 4. *HPLC-FLD chromatograms of the purified toxins from the viscera in A.floridus collected on the reef (site B) of Ishigaki Island*

(I): GTX stds. (a: GTX4, b:GTX1, c:GTX3, d:GTX2), (II) :STX stds. (e:hyneoSTX, f:neoSTX, g:hySTX, h:dcSTX, i:STX), (III) and (IV) : GTX and STX analysis of purified toxins from the viscera

For GTX analysis (Figure 4-I, III), the one main peak was identified as GTX2. In addition to this, GTX4, 1 and 3 were detected as trace components on the chromatogram. In STXs analysis (Figure 4-II, IV), the toxin revealed three peaks with the same retention times of hydroxyneosaxitoxin (hyneoSTX), neoSTX and STX, respectively. Toxin profiles of the viscera of *A.floridus* from site A and B in June, 2007 are illustrated in Fig.5.

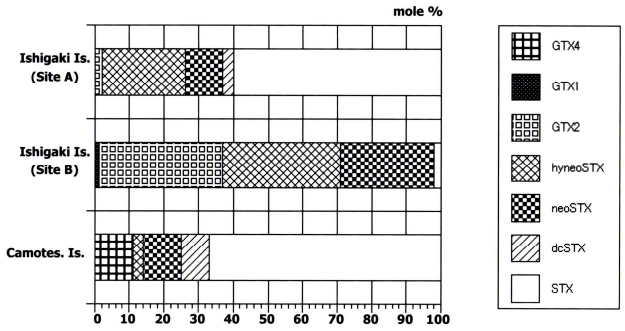

Figure 5. *Comparison with the toxin profiles of A.floridus collected on reefs of Ishigaki Island and Camotes Island, Cebu Province, Philippines*

In site A, relative abundances (mole %) of carbamoyl-*N*-hydroxyneosaxitoxin (hyneoSTX), neosaxitoxin (neoSTX), and saxitoxin (STX) were high (98%), and only 2% of gonyautoxin 2(GTX2) were contained in addition to similar amounts (3%) of decarbamoylsaxitoxin (dcSTX). The viscera of *A.floridus* from site B in the same day possessed GTX2 (36%) and STX group (63 %) predominantly, and only 1% of GTX1 was contained in addition to similar amount (2%) of STX. Although the distance between site A and B were extremely near each other, there seemed to be marked narrow regionality of toxicity and toxin profiles. It was also reported that *A.floridus* on the reefs near Kabira Bay, Ishigaki Is. possessed STX predominantly over the other STX derivatives with no detectable amount of GTXs [18]. Such variation may be explainable, taking the spotty distribution of the causative agents into consideration. The reef on right-side is rocky, while the reef on left-side is bound in algae, especially, such as red algae and calcareous red alga. Kotaki et al., detected low levels of PSP in a calcareous alga *Jania* sp. and presumed its involvement in the intoxication of xanthid crabs the primary source of paralytic shellfish toxins in crabs and gastropods on the reef [25]. Later, TTX was also detected in this alga [26].

On the other hand, Saisho et al., reported the eating habits of the xanthid crabs, as examined from the composition of their stomach contents, irrespective of their PSP toxicity levels [27]. Judging from their results, the PSP containing highly toxic xanthid crabs such as *A.floridus* and *Z.aeneus* was postulated to be rather omnivorous (not planktonic) feeder than herbivorous. Actually, from stomach contents in *A.floridus* from Ishigaki Is. in June, 1979 *Hypnea* sp., sand, shell, animal tissues, poriferans and fish fragments with no parts of *Jania* sp. were detected. By the way, in the semi purified toxins from the viscera of Camotes sample, relative abundances (mole %) of neoSTX, dcSTX and STX were rather high (89%), and 11% of GTX4 were contained in addition to smaller amounts (3%) of hyneoSTX (Figure 5). PSP compositions were almost the same as that of Ishigaki specimens, but in HPLC-FLD analysis of TTX, trace amount of TTX was detected (data not shown).

Furthermore, ion-monitored mass chromatograms of TMS derivatives of alkali-hydrolyzed toxin and authentic TTX in the GC-MS method are shown in Figure 6. Mass fragment ion peaks at *m/z* 376, 392 and 407, which are characteristic of the quinazoline skeleton, appeared at almost the same retention times of 15.12 and 15.16 min, respectively, and along with TMS-C9 base derived from authentic TTX with a retention time of 15.14 min. The crab toxin and standard TTX had the same mass spectra with mass fragment ions peaks at *m/z* 407 (molecular peak), 392 (base peak) and 376. Therefore, from the results of HPLC-FLD and GC-MS analysis, it can be concluded that the toxin in *A.floridus* from Camotes Is. is a mixture of PSP and a trace amount of TTX.

As seen in our present study, in the toxic crabs living on reefs of Ishigaki Is., STX was predominant. However, *A.floridus* inhabiting Kojima, a small islet near site A and B (Fig.1), as seen in the Pacific coasts of Japan Proper, had TTX and/or its derivatives of as major toxins[28].

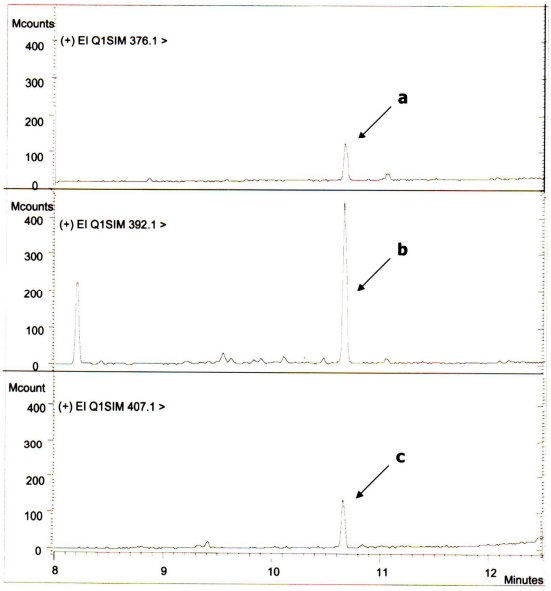

Figure 6. *GC-MS analysis of TMS derivative of C9 base in paralytic toxins from A. floridus collected from Camotes Island, Cebu Province, Philippines a, m/z = 376; b, m/z = 392; and c, m/z = 407*

In this connection, it was previously reported that TTX is the major toxin, and GTX 1 and, 3 are minor toxins in *L. pictor* (xanthid crab from Taiwan) [29]. In contrast, in crabs from Negros Island, Philippines, PTX was found to be the predominant toxin [30, 31].

On the other hand, TTX and PSP are the major and minor toxins, respectively, in *A.floridus* inhabiting Miura Peninsula, Kanagawa Prefecture, Japan [9], which differs from the same species used in this study. *A.floridus* specimen from Fiji Island was found to have, STX and STX derivatives as major toxins [24]. This indicated that the crab toxin might be of exogenous rather than endogenous origin. Through a complex system of trophic interrelationships, non filter-feeding organisms can also be exposed to PSP and/or TTX and thus accumulate and play a role as vectors in marine food web. Transport and accumulation of toxins in food chains are a common phenomenon, particularly in marine biota. The origin of neurotoxins in toxic xanthid crabs may be from toxic lower strata invertebrates. In order to elucidate the diet of *A.floridus* from site A, B and the Camotes Is., microscopic examination of stomach contents of the species is needed. It was reported that PSP-containing *A.floridus* maintains a fairly high toxicity level for a long period when fed nontoxic diets (Noguchi et al., personal communication). This observation may suggest that PSP in this toxic crab mentioned above be endogenous. Oikawa [32] showed the presence of PSP toxins in the viscera of the edible shore crab *Telmessus acutidens*. It was also revealed that the total toxicity in the crab viscera increased linearly with the amount of toxic mussels the crabs ingested by feeding experiments [33].

Further studies are now in progress to elucidate the associated mechanism of toxicity. The accumulation mechanism or exact metabolic pathway of the toxins in xanthid crabs remains to be elucidated. As the screening of alga in the reef in Ishigaki island and Camotes Island are now on progress, the results will be published in elsewhere.

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