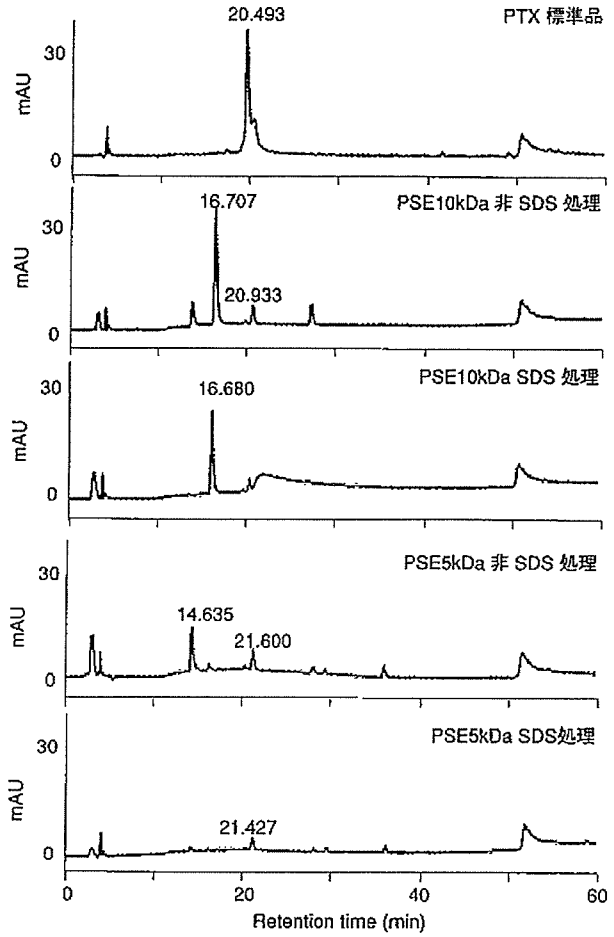

図２－ａ　各種フィルターろ過による PTX の HPLC 分析結果

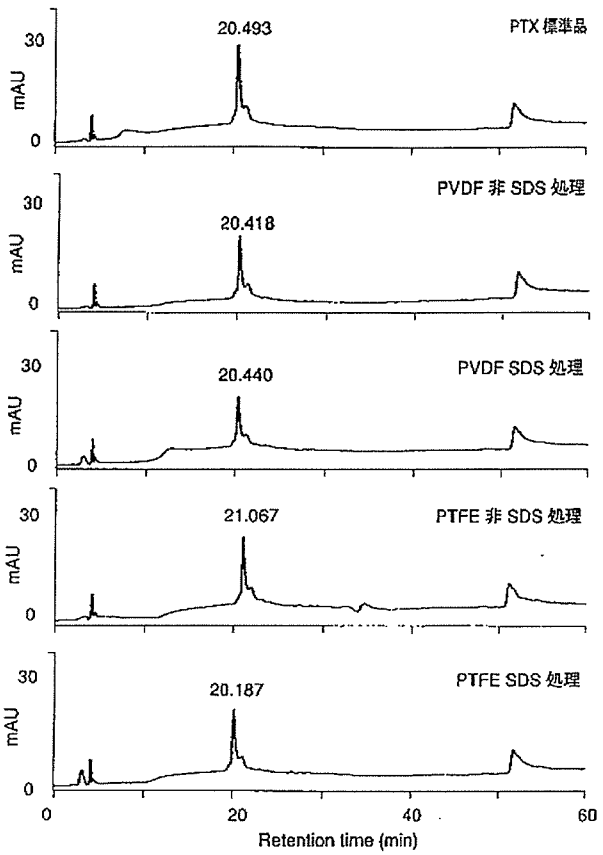

図２－ｂ　各種フィルターろ過による PTX の HPLC 分析結果

PTX をほぼ100％回収でき，成分の変換も認められなかったため，機器分析試料の調製のための前処理に有効であると思われた。

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Detection of tetrodotoxin (TTX) from two copepods infecting the grass puffer *Takifugu niphobles*: TTX attracting the parasites?

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Abstract

In May 2002, two parasitic copepods, *Pseudocaligus fugu* and *Taeniacanthus* sp., were collected from the body surface and gill of the grass puffer *Takifugu niphobles*, respectively, in Takehara city, Hiroshima Prefecture, faced with Seto Inland Sea located in the western part of Japan. To them was added 5 ml of 0.1% acetic acid, then the suspension was subjected to ultrasonic disruption with an ultrasonicator for 10 min. The resulting mixture was heated in a boiling water bath for 10 min, and then centrifuged. The supernatant was concentrated under reduced pressure, and loaded on to a Sep-Pak plus C18 Environmental Cartridge (Waters). The unbound fraction was analyzed by HPLC and gas chromatography–mass spectrometry (GC–MS) for tetrodotoxin (TTX). It was rather unexpectedly revealed from these results that this fraction was comprised of TTX and its analogues. As far as we know, this is the first record to show the existence of TTX in the copepods. In addition, relationships between the more and less than the average number of the two parasites and the toxicity of its skin mucus of the host were examined by student's *t*-test. In *P. fugu*, the average number per host was 13.9, and those are 520.7 ($n = 9$) and 269.0 MU/g ($n = 22$), respectively. A highly significant difference between them was detected at *p*-value 0.0011. In contrast, as for *Taeniacanthus* sp., the average number was 2.7, and those were 338.0 ($n = 14$) and 345.5 MU/g ($n = 17$), respectively. No significant difference was detected in *Taeniacanthus* sp. The high host-specificity of *P. fugu* on the toxic puffer and the present bioassay of its skin mucus suggest a possibility that TTXs may attract the parasite.

Keywords: Tetrodotoxin; Skin mucus; Parasitic copepod; *Pseudocaligus fugu*; High performance liquid chromatography; Gas chromatography–mass spectrometry

1. Introduction

Tetrodotoxin (TTX), known as puffer fish toxins, is one of the most potent nonpeptidic neurotoxin

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because of its frequent involvement in fatal food poisoning, its unique chemical structure, and its specific action of blocking sodium channels of excitable membranes (Colquhon et al., 1972; Evans 1972; Narahashi 2001). The toxin derives its name from the pufferfish family *Tetraodontidae*, but past studies have revealed its wide distribution in both terrestrial and marine animal kingdoms such as vertebrate species inclusive of pufferfish, goby, newt and frog, and invertebrates of octopus, gastropod mollusk, crab, starfish, and nemertean and turbellarian (Ali et al., 1990; Hwang et al., 1994; Mebs et al., 1995; Mahmud et al., 1999; Asakawa et al., 2000, 2003; Mebs, 2001; Miyazawa and Noguchi, 2001; Hanifin et al., 2002). There is no phylogenic relationship among these TTX-containing animals. In many species of the family *Tetraodontidae* such as the grass puffer *Takifugu niphobles*, TTX are also well known to be contained in the skin, skin mucus except internal organs. The external body surface plays a very important role in the prevention of pathogen invasion, especially in fish, which continuously exposed to an aquatic environment. In addition to the mechanical barrier of scales, the skin surface of fish is protected by the secretion of mucus. Fish skin mucus contains many molecules that may act as defense factors (Ingram, 1980; Alexander and Ingram, 1992). However, the biological adaptive values of the presence of TTX in these organisms are not evidently clarified. In the recent studies on toxic puffers, as possible physiological roles of TTX except a defense agent, functions as sex pheromone (Matsumura, 1995) and enhancing substances of the immune system are reported (Arakawa, 2002). On the other hand, it is also known that there are a number of ectoparasites infecting toxic puffers (Ogawa, 1991, Hirazawa et al., 2001, Okabe, 2003). Ikeda et al. (2006) have first revealed with an immunoenzymatic technique that TTX is accumulated in the whole parts of an ectoparasitic copepod *Pseudocaligus fugu* infecting the grass puffer *T. niphobles* except for its female reproductive system and eggs, suggesting that TTX might have been accumulated by their feeding on the skin and mucus of the host.

These circumstances prompted us to investigate TTX and its derivatives in parasitic copepod attaching to the grass puffer. The present paper reports to confirm the existence of them in two parasitic copepods, *P. fugu* and *Taeniacanthus* sp., attaching to the host *T. niphobles* collected from Seto Inland Sea, the western part of Japan in May 2002, using high performance liquid chromatography (HPLC)-fluorometric system and gas chromatography–mass spectrometry (GC–MS). In addition, relationships between the number of these two parasitic copepods infecting *T. niphobles* and the toxicity of the skin mucus of *T. niphobles* are also investigated.

2. Materials and methods

2.1. Parasitic copepods and skin mucus

In May 2002, specimens of the grass puffer *T. niphobles* were collected by fishing in front of the Takehara Marine Science Station, Setouchi Field Science Center in the Seto Inland Sea, Hiroshima University, Hiroshima Prefecture, Japan (Fig. 1). With fine forceps, all live parasitic copepods *P. fugu* (preadults and adults) and *Taeniacanthus* sp. (copepodids and adults) were carefully picked up from the body surface and gill of the host, respectively. Live copepods were kept in glass beakers filled with filtered (Whatman GF/C) seawater at 20 °C for 2 or 3 days to remove contamination of organization and mucus of the host and gut contents of the parasites. After that copepods were kept frozen at −20 °C in a freezer.

The host *T. niphobles* were subjected to a light wiping of the whole body surface with gauzes (designated "handling stimulus"). The wiped gauzes were kept frozen at −20 °C in a freezer.

2.2. Assay of toxicity

Toxicity of the skin mucus on the body surface of *T. niphobles* was estimated by the official Japanese method for TTX (Kawabata, 1978). The toxicity was expressed in mouse unit (MU), in which one MU was defined here as the amount of toxin, which killed 19–21 g ddY strain male mice in 30 min after intraperitoneal administration.

2.3. Purification of toxin

Collected specimens of parasitic copepods *P. fugu* and *Taeniacanthus* sp. weighed 0.042 g ($n = 30$) and 0.027 g ($n = 50$), respectively, on a wet basis. To them was added to 5 ml of 0.1% acetic acid, then the suspension was subjected to ultrasonic disruption with an ultrasonicator for 10 min. The resulting mixture was heated in a boiling water bath for 10 min, cooled to room temperature, and then

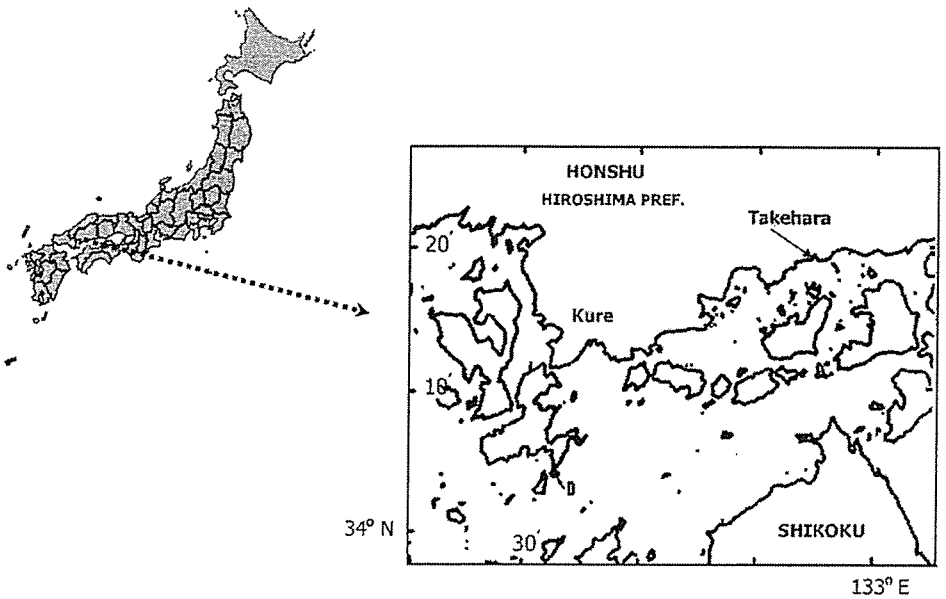

Fig. 1. Map showing the sampling location of Takaehara in Hiroshima Prefecture, Japan.

centrifuged at 3000 × *g* for 10 min. The supernatant was concentrated under reduced pressure, and loaded on to a Sep-Pak plus C18 Environmental Cartridge (Waters). The unbound portion was collected and concentrated to dryness. The resulting solid was dissolved in a small amount of water, and injected into the HPLC-fluorometric system for the analysis of TTX and its related toxins (Asakawa et al., 2000, 2003). The wiped gauzes were ultrasonicated in 10 ml of 0.1 ml of acetic acid for 3 min and centrifuged at 3000 × *g* for 10 min. The supernatant was concentrated under reduced pressure and filtered with an ultra-filtration kit (Ultra-free C3, Millipore), whose cut-off limit was 3000 Da. The obtained extracts were described as the "secreted toxin solution", or simply the "secretion", and then analyzed for the same HPLC system mentioned above.

As authentic toxins, a mixture of TTX and anhydrotetrodotoxin (anhTTX) was prepared from the ovaries of puffer *T. vermicularis* essentially by the method of Goto et al. (1965). It contained also several percent of 4-epiTTX.

2.4. Gas chromatography–mass spectrometry

A small amount of the extract from the parasitic copepods and authentic TTX were degraded with alkali. The degradation product was trimethylsilylated, and then analyzed for 2-amino-6-hydroxymethyl-8-hydroxyquninazoline (C9 base) by GC–MS according to the previously described procedure (Narita et al., 1987; Asakawa et al., 2000, 2003). Prepared trimethylsilyl (TMS) derivatives were submitted to a gas chromatograph (Hewlett Packard, HP-5890-II) equipped with a mass spectrometer (AutoSpec, Micromass Inc., UK). A column (φ0.25 × 250 cm) of UB-5 (GL Sci., Japan) was used and the temperature was raised from 180 to 250 °C at a rate of 5 °C/min. The ionization voltage was 70 eV and the ion source temperature was kept at 200 °C. Scanning was carried out in the mass range of *m/z* 40–600 at 3 s interval.

3. Results and discussion

The HPLC patterns of the TTX group contained in 0.1% acetic acid extracts from the two species of parasitic copepods and mucus of the puffer fish are shown in Fig. 2. The analysis of skin mucus of T. niphobles clearly revealed three peaks containing of TTX, 4-epiTTX and anhTTX (Fig. 2B) in comparison with the standard TTXs (Fig. 2A). The analysis of the extract of *P. fugu* revealed four peaks, of which three could be identified clearly as TTX, 4-epiTTX and anhTTX. The fourth component was not identified (Fig. 2C). In the HPLC analysis of the extract of *Taeniacanthus* sp., TTX was clearly detected along with the same retention time of the standard TTX (Fig. 2D). The ion-monitored mass chromatograms of TMS derivatives of alkali-hydrolyzed extracts of the two parasitic copepods *P. fugu* and *Taeniacanthus* sp. are

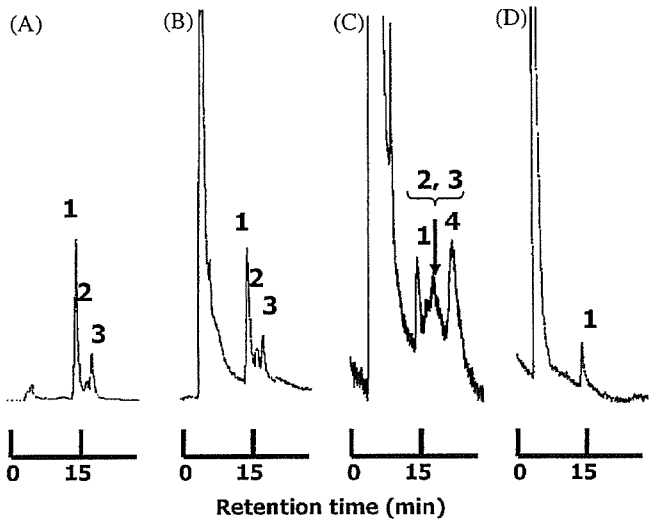

Fig. 2. HPLC analysis of TTX and its derivatives contained in the parasitic copepods and skin mucus of *Takifugu niphobles*. (A) TTXs standard: (1–3) tetrodotoxin, 4-epi-tetrodotoxin, anhydrotetrodotoxin. (B) Skin mucus of *Takifugu niphobles*; (C) *Pseudocaligus fugu*; (D) *Taeniacanthus* sp.

depicted in Fig. 3. Mass fragment ion peaks at *m/z* 376, 392, and 407, which are characteristic of the quinazoline skeleton (C9 base), appeared at the same retention times (*P. fugu* 10.29 min; *Taeniacanthus* sp. 10.30 min), respectively, along with TMS-C9 base derived from authentic TTX with a retention time of 10.26 min. All of these peaks from alkali-hydrolyzed extracts of these two parasitic copepods and authentic TTX revealed essentially the same mass spectra which were featured by fragment ions at *m/z* 407 (molecular peak), 392 (base peak), 376, 320, 318 and 230 (data not shown).

It can be concluded from the results of HPLC and GC–MS analysis that the extracts from the parasitic copepods contained TTX and its derivatives. As far as we know, this is the first report concerning the presence of TTX and its derivatives in the bodies of copepods. In this connection, it was indicated that the transmission of paralytic shellfish poison (PSP), which is the same Na^+ channel blocker as TTX, of the dinoflagellate toxins through herbivorous zooplankton as vectors to higher trophic levels and that they can reach sufficient levels in zooplankters to cause fish and its larvae kill(White, 1979, 1980, 1981; White et al., 1989). It is reported that planktonic copepods, *Acartia tonsa* and *Eurytemora herdmani* accumulate PSP by feeding on toxic dinoflagllates (Teegarden and Cembella, 1996). Hence not only parasitic but also planktonic copepods generally have a capability to be resistant to Na^+ channel blockers such as PSP and TTX.

On the other hand, some intestinal bacteria of TTX-bearing animals were demonstrated to produce TTX (Miyazawa and Noguchi, 2001). It suggests that TTX-bearers become toxic through the food chain in which TTX is transferred from lower to higher strata animals. This, along with the phylogenetically irregular occurrence of TTX, suggests that some microorganisms could be true producers of this toxin.

Relationships between the number of the two parasitic copepods on *T. niphobles* and the toxicity of its skin mucus of *T. niphobles* are depicted in Fig. 4. The numbers of *P. fugu* and *Taeniacanthus* sp. per host ranged from 0 to 94 individuals (average $\pm$ standard deviation $= 13.9 \pm 22.6$) and from 0 to 8 individuals (2.7 ± 2.8), respectively. The toxicity of the skin mucus of *T. niphobles* had a range of 108.5–1070.4 MU/g (average $\pm$ standard deviation $= 342.1 \pm 208.2$). Toxicity was detected from the skin mucus of all the hosts. Some evidence that may elucidate the physiological significance of this toxin in puffers has been recently reported. Saito et al. (1985) observed that puffers released large amounts of TTX from the skin when lightly wiped with gauze, and suggested that TTX in the mucus layer covering the integument of them may act as self-defense agent against predators. Kodama et al. (1985) found a similar phenomenon with a puffer stimulated by electric shock. In this connection, Kodama et al. (1986) also reported that unique exocrine glands or gland-like structures were found in the skin of several species of the puffer genus *Takifugu*. The glands of *T. pardalis* and *T. vermiculare porphyreum* consisted only of secretary cells with large vacuole.

Relationships between the more and less than the average number of the two parasites and the toxicity of its skin mucus of the host were examined by student's *t*-test (Table 1). In *P. fugu*, the average number per host was 13.9, and those are 520.7 ($n = 9$) and 269.0 MU/g ($n = 22$), respectively. A highly significant difference between them was detected at *p*-value 0.0011. In contrast, as for *Taeniacanthus* sp., the average number was 2.7, and those were 338.0 ($n = 14$) and 345.5 MU/g ($n = 17$), respectively. No significant difference was detected in *Taeniacanthus* sp.

The present study clearly coincides with the presence of TTX in the body of the parasitic copepod *P. fugu* on *T. pardalis*, which has been revealed by an immunoenzymatic technique (Ikeda et al., 2006). The fish ectoparasitic copepods are

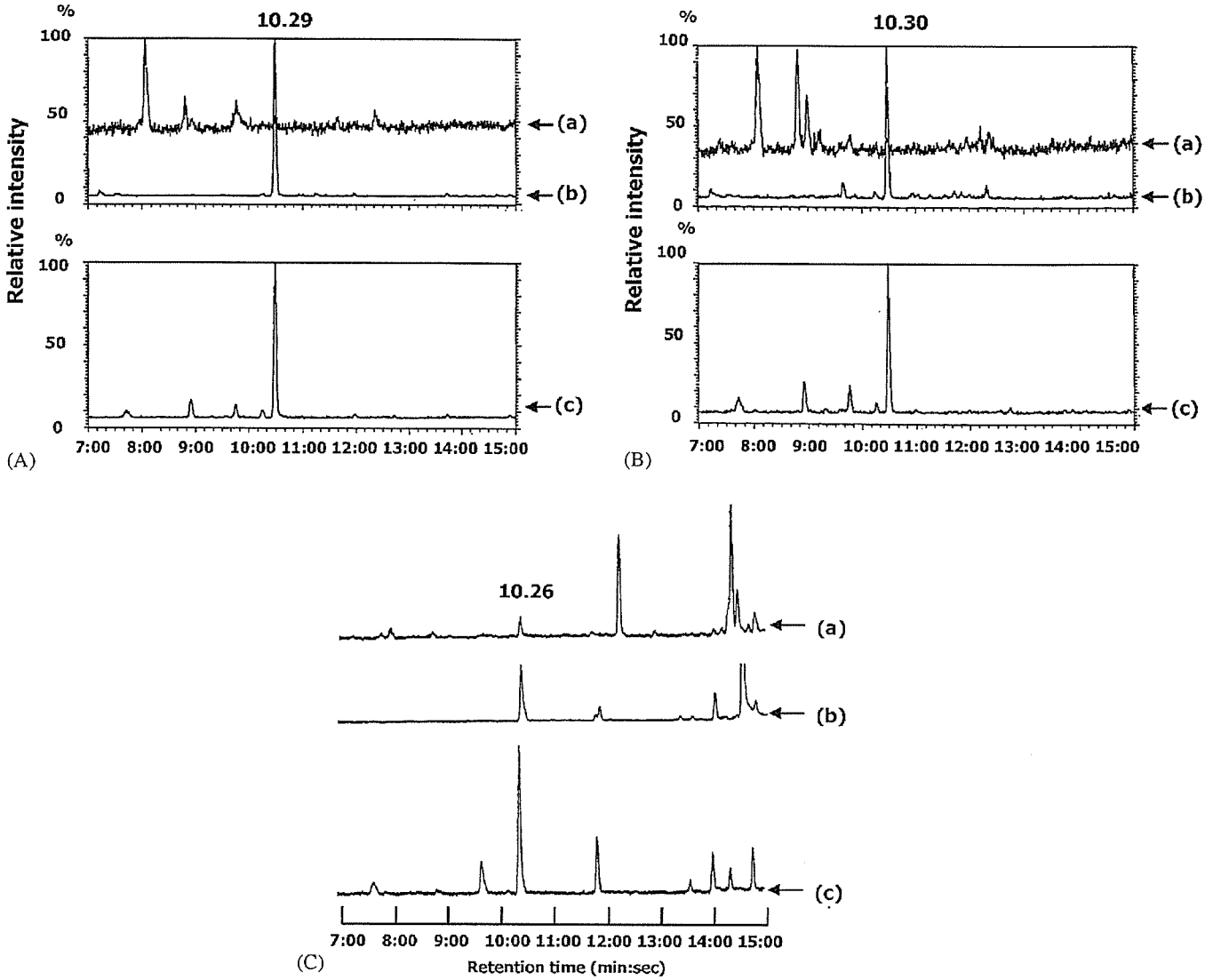

Fig. 3. Ion-monitored chromatograms of the trimethylsilyl derivative of the C9 base from the toxins contained in the parasitic copepods. (A) *Pseudocaligus fugu*; (B) *Taeniacanthus* sp.; (C) TTX standards (a–c); $m/z = 376, 407, 392$.

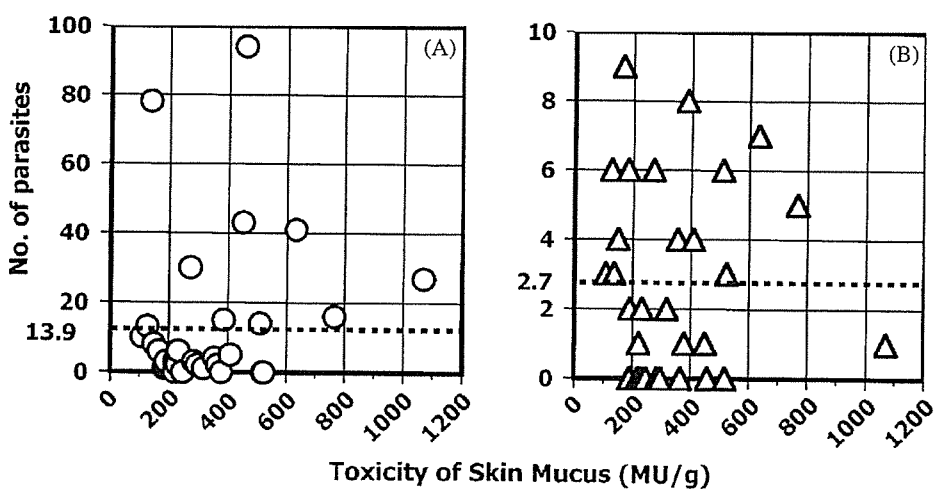

Fig. 4. Relationships between toxicity of mucus on the skin of the grass puffer *Takifugu niphobles* and the number of parasites on hosts. Dotted line in these figures shows average number of parasites. (A) *Pseudocaligus fugu*; (B) *Taeniacanthus* sp.

Table 1
Two groups of comparison divided by the average of parasites by student's *t*-test

Copepods	Over average		Under average		*p*-value
	No. of samples of *T. niphobles*	Mean toxicity (MU/g)	No. of samples of *T. niphobles*	Mean toxicity (MU/g)	
Pseudocaligus fugu	$n = 9$	520.7	$n = 22$	269.0	0.0011
Taeniacanthus sp.	$n = 14$	338.0	$n = 17$	345.5	0.9222

considered to feed on mucus, tissue and blood of host (Kabata, 1984). Since TTX and its derivatives were detected from both the host mucus and *P. fugu* in the present study, this skin parasite seems to have taken the mucus as food and accumulated TTXs in the body. The life cycle of the family Caligidae accommodating *P. fugu* is well investigated, which consists of two free-swimming naupliar stages, a single infective copepodid stage, four to six chalimus stages, one to two preadults (without molt), and one adult (Ho and Lin, 2005). The life cycle of *P. fugu* is incompletely addressed by us, but there are, at least, two naupliar and a single copepodid stages as free-swimming stages (Okabe, 2003). Since TTX is not accumulated in the ovary and eggs of the adult female of *P. fugu* (Ikeda et al., 2006), acquisition of this toxin seems to occur through feeding on the mucus and tissues of the host from the chalimus to the adult after attachment of the infective copepodid stage on it. The present HPLC result suggests that the gill parasite *Taeniacanthus* sp. has a different composition of toxin in the body. This may be explained by the following reasons: (1) toxic composition differs between the gill and skin mucus and/or (2) chemical conversion occurs in the body of the parasite.

The skin parasite *P. fugu* is found exclusively from the toxic puffer such as *T. niphobles*, *T. oblongus*, *T. pardalis*, and *T. poecilonotus* (Ho and Lin, 2005; Ikeda et al., 2006). The high host-specificity of *P. fugu* on the TTX-bearing puffer and the present bioassay strongly suggest a possibility that TTX may play a role in attracting the infective copepodid stage. In addition, preadults and adults of caligids detaching from the host can swim freely in water column (Ohtsuka, unpublished data). This may be only accidental and/or for active host switching. Also in that case, free-swimming preadults and adults of copepods of *P. fugu* may be re-attracted by TTX released from the host. Behavioral reaction of *P. fugu* to TTX will be observed in a laboratory in the future. However, the biological meanings of accumulation of TTX in the body and resistance mechanism against TTXs for the copepods are still unknown.

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