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NOTE

Trace amounts of saxitoxins in the viscera of chum salmon *Oncorhynchus keta*

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ABSTRACT: Low levels of saxitoxins and tetrodotoxin were found in the viscera of adult chum salmon *Oncorhynchus keta* returning to spawn. The nucleic magnetic resonance and mass spectrum analyses as well as several kinds of chromatographies clearly showed that the purified toxins were saxitoxin and neosaxitoxin. Tetrodotoxin, which was detected by high performance liquid chromatography analysis, could not be isolated. These findings show that saxitoxins and tetrodotoxin are more widely distributed in aquatic organisms than previously thought.

KEY WORDS: Saxitoxin · Tetrodotoxin · Chum salmon · *Oncorhynchus keta*

In a previous paper (Sato et al. 1993), we observed sodium channel blocking activity in extracts of various common aquatic species, though the activity was very low. When the toxin with this activity was partially purified and analyzed by high performance liquid chromatography (HPLC) (Yotsu et al. 1989, Oshima 1995), saxitoxins (STXs) and tetrodotoxin (TTX) were detected (Sato et al. 1993). STXs are well-known dinoflagellate toxins which cause paralytic shellfish poisoning (Schantz 1986). STXs occur in various aquatic organisms other than dinoflagellates, including xanthid crabs (Noguchi et al. 1969), horseshoe crabs (Fusetani et al. 1982), marine snails (Kotaki et al. 1981) and puffers (Kodama et al. 1983, Sato et al. 1997). On the other hand, TTX is a neurotoxin which binds to the same receptor as STXs (Kao 1966). TTX is a well-known puffer toxin, but is known to occur in other aquatic species (Mosher & Fuhrman 1984). Species which possess either STXs or TTX are now known to possess both toxins, that is, TTX has been detected in xanthid crabs (Noguchi et al. 1983) and horseshoe crabs (Kungsuwan et al. 1987, Saitanu et al. 1988), while STXs have been found in puffers (Kodama et al. 1983, Sato et al. 1997). Recently, TTX was detected in *Alexandrium tamarense*, a representative dinoflagellate which produces STXs (Kodama et al. 1996). These findings suggest that TTX and STXs are widely distributed in natural ecosystems in certain species. By contrast, our previous results show that TTX and STXs are not specific toxins detected in selected species, but are distributed widely among common aquatic species at low levels. If true, this becomes important from the standpoint of the origin and the biological impact of these toxins. In the present study, we chose chum salmon *Oncorhynchus keta* because TTX, STX and neosaxitoxin (neoSTX) have been detected in this species by HPLC analysis (Sato et al. 1993); we purified sodium channel blocking toxins from the viscera to a highly purified state. We report here the occurrence of STX and neoSTX in the viscera of chum salmon with additional and more convincing chemical evidence.

Viscera were collected from chum salmon *Oncorhynchus keta* caught at the river mouth of Sakari river, Iwate, Japan, during November and December, the homing period of the salmon, in 1995. A total of 62 kg of viscera was collected. Once near the mouth of the stream, more than 90% of the chum salmon which return to the parent river do not feed (Ueno 1993), and all stomachs in the viscera collected were empty.

The toxin in the viscera was extracted with 2 volumes of boiling 0.1 M acetic acid and purified. During the purification process, the toxin was monitored by mouse bioassay and the toxin amount was expressed in mouse units (MU), where 1 MU will kill a 20 g male mouse in 15 min. About 6000 MU of toxin was obtained. After an activated charcoal treatment (Kotaki et al. 1981), the crude toxin was applied to a column of Amberlite CG50 with mesh size of 100 to 200 (Na^+ form, 5 × 70 cm). The column was washed with a suffi-

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cient volume of water, and the substances adsorbed to Amberlite CG50 were eluted with 4000 ml of 1 M sodium acetate and 1 M acetic acid, successively. In total T, 2590 MU of the toxin was recovered in the 1 M acetic acid fraction. A part of the toxin with more negative charge was eluted in the 1 M sodium acetate fraction. However, we were unable to extract toxins from this fraction because of difficulties associated with purification of a small amount of toxins in a high concentration of sodium acetate solution. The toxin in the acetic acid fraction was further purified by repeated chromatography on Bio-Gel P-2 column (200 to 400 mesh, 5 × 70 cm) equilibrated with 0.03 M acetic acid. The 2450 MU of toxin recovered was then subjected to Bio-Rex 70 column chromatography (H^+ form, −400 mesh, 1 × 90 cm) using linear gradient elution from 100 ml of distilled water to 100 ml of 1 M acetic acid. Two toxic fractions (toxins 1 and 2) were obtained. Respective toxic fractions were further purified by HPLC on a Hitachi-gel 3011C column (4.6 × 250 mm). In total, 1240 MU of toxin 1 and 1010 MU of toxin 2 were obtained in a highly purified state. In the HPLC-fluorometric analysis for STXs (Oshima 1995), by thin layer chromatography (Buckley et al. 1976) and by electrophoresis (Fallon & Shimizu 1977), toxins 1 and 2 were indistinguishable from standards of STX and neoSTX (data not shown). The nucleic magnetic resonance spectra of toxins 1 and 2 measured in D_2O coincide with those of standard STX and neoSTX measured under the same conditions (Table 1). Positive fast atom bombardment mass spectra of toxins 1 and 2 measured in a glycerol matrix showed ion peaks at m/z = 300 and 316 which correspond to respective pseudomolecular ion peaks of STX and neoSTX. These data clearly show that STX and neoSTX are present in the viscera of chum salmon, and support our previous finding that STXs are not restricted to the selected species, but occur in a variety of aquatic organisms at low levels. We could not isolate TTX which we detected in the viscera of chum salmon by HPLC analysis (Sato et al. 1993). During the purification process, about half the toxin was lost in the Amberlite CG50 chromatography. Probably TTX was lost in the 1 M sodium acetate fraction in the chromatography because of its weaker positive charge than STX and neoSTX.

Table 1. Comparison of nucleic magnetic resonance spectra (^{1}H-NMR) of toxin 1 and toxin 2 isolated from the viscera of chum salmon *Oncorhynchus keta* with those of standard saxitoxin and neosaxitoxin. s: singlet; d: doublet; dd: double doublet; m: multiplet. Neosaxitoxin data from Shimizu et al. (1978)

Carbon no.	Chemical shift (coupling constant) Toxin 1	Toxin 2	Saxitoxin	Neosaxitoxin
H5	4.68 s	4.78 s	4.66 s	4.74 s
H6	3.78 m	4.06 dd (6,6.5)	3.77 m	3.96 dd (6,6.5)
H10	3.75 m	3.76 dd (11,10)	3.73 m	3.67 d (10)
	3.55 m	3.54 m	3.52 m	3.46 d (10)
H11	2.33 m	2.35 m	2.30 m	2.13 m
H13	4.28 dd (11,9)	4.38 dd (12,6)	4.22 dd (11,9)	4.41 dd (12,6)
	3.98 dd (11,6)	4.18 dd (12,6)	3.94 dd (11,9)	4.22 dd (12,6.5)

STXs are produced by several species of dinoflagellates (Schantz 1986). During a bloom of these dinoflagellates, bivalves become toxic by accumulating STXs from dinoflagellates via the food chain (Schantz 1986). However, the origin of STXs in species other than those which are associated with dinoflagellates is unknown. A low level of STXs is produced by bacteria isolated from toxic dinoflagellates such as *Alexandrium tamarense* (Kodama et al. 1988, Doucette & Trick 1995, Gallacher et al. 1997). Bacteria known to produce STXs may be widely distributed in marine ecosystems (Kodama et al. 1990, Sakamoto et al. 1992, Levasseur et al. 1996). In a previous paper (Sato et al. 1993), no significant difference in toxin potency was observed from viscera of various common species representing diverse phyla, indicating that the toxin was not accumulated at higher levels in the food web. The observation that all stomachs in the viscera used in the present study were empty supports our contention that toxins do not originate from diet, although regurgitation may have occurred. These facts suggest that a low level of STXs in viscera from common aquatic species may be due to STXs-producing bacteria from the intestinal bacterial flora occurring naturally.

On the other hand, TTX is a well-known toxin produced by bacteria (Noguchi et al. 1986, Yasumoto et al. 1986). TTX-producing bacteria are reported to occur widely in marine ecosystems (Simidu et al. 1987). For example, Noguchi et al. (1987) report that TTX is produced by *Vibrio alginolyticus*, which is known to be distributed widely in the oceans and in the intestinal bacterial flora of marine species. These facts suggest that most marine organisms are exposed to TTX-producing bacteria. Although we lost the TTX-like toxin during the purification procedures in the present study, it is possible that a low level of TTX from environmental bacteria may occur in common aquatic species together with STXs. It has been suggested that the selected organisms which possess high amounts of TTX and STXs accumulate these toxins via the food web (Yasumoto & Yotsu 1992). Species which possess both STXs and TTX are highly resistant to these toxins (Saito et al. 1985, Daigo et al. 1988), and this resistance may be associated with their ability to accumulate a high level of these toxins. Our results indicate that these toxins

occur in common aquatic species at low levels. Apparently, these may support a dietary mechanism by which the selected organisms such as puffer accumulate high amounts of toxins. However, puffer show a remarkable individual difference in toxicity (Kodama et al. 1984), though no significant difference was observed in toxin potency from viscera of various common species which could be in the diet of puffer (Sato et al. 1993). Therefore, puffer seem to accumulate high amounts of toxins by an unknown mechanism other than food-web interaction. Previously, we reported that toxic specimens of puffer are infected by bacteria which produce a TTX-like substance, while no such infection was observed in nontoxic specimens (Kodama et al. 1995). Accumulation of high amounts of TTX and STXs in the selected organisms seems to be due to the infection of these toxin-producing bacteria.

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