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Tetrodotoxin-binding proteins isolated from five species of toxic gastropods

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Abstract

Toxic gastropods *Polinices didamy*, *Natica lineata*, *Oliva miniacea*, *O. mustelina* and *O. hirasei* are tetrodotoxin (TTX) bearing animals, which accumulate TTX in their muscle and digestive gland. Analysis by gel filtration on Sepharose CL-6B revealed that 0.05 M NaCl extracts of the muscle of five species of toxic gastropods contained TTX-binding high molecular weight substances (HMWS) (1500–2000 kDa). The TTX-binding capacities of those HMWS were 0.12, 0.62, 0.45, 0.28 and 0.35 MU/mg protein, respectively, but those HMWS had no neutralising effect on TTX or paralytic shellfish poison. The HMWS of the five toxic gastropods could be hydrolyzed with HCl and protease at 37 °C, pH 7.4, but not with ribonuclease T2, deoxyribonuclease I or α-amylase. After purifying the TTX-binding protein of *N. lineata* by Q Fast-Flow strong anion exchanger and then BioSep-SEC-S 2000, the TTX-binding capacity increased to 3.5 MU/mg and 4.2 MU/mg protein, respectively. The TTX-binding capacity of *N. lineata* HMWS had no obvious seasonal variation. The molecular weight of TTX-binding protein of *N. lineata* was estimated to be about 434 kDa, while it comprised two subunits with molecular weights of about 272 kDa and 47 kDa, respectively, under SDS–PAGE.

Keywords: Tetrodotoxin (TTX); TTX-binding protein; Toxic gastropod; *Natica lineata*

1. Introduction

Tetrodotoxin (TTX) is a guanidinium toxin that blocks voltage-dependent sodium channels involved in the generation of an action potential. TTX is located in glands in the epidermis of the skin, ovary and liver in puffer fish, and also in the secretary glands of skin in newt (Mahmud et al., 2003). It is curious how these TTX-bearing animals can accumulate toxin in their body without killing themselves. Therefore it is proposed that TTX is wrapped in a particular protein, and does not bind directly to the target side-sodium channel, and hence does not induce intoxication. TTX-bearing animals are in general much more resistant to the lethal effect of TTX than animals having no TTX. For instance, the toxic xanthid crabs *Zosimus aeneus* and *Atergatis floridus* showed an extremely high resistance to TTX administrated intrahaemocoelically (Nagashima, Ohgoe, Yamamoto, Shimakura, & Shiomi, 1998). Nagashima, Yamamoto, Shimakura, and Shiomi (2002) reported that the shore crab *Hemigrapsus sanguineus* contained a soluble protein that could bind TTX. Its molecular weight was estimated to be about 400 kDa, and comprised two subunits with molecular weights of about 72 kDa and 82 kDa, respectively. A TTX-binding protein has been purified from the plasma of the puffer fish *Takifugu niphobles*, and its molecular weight estimated to be about 116 kDa by SDS–PAGE. The terminal amino acid sequence of TTX-binding protein of *T. niphobles*

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revealed that the protein had no homology to any other protein in the data base (Matsui, Yamamori, Furukawa, & Kono, 2000). Yamashita-Yotsu et al. (2001) purified a novel soluble glycoprotein that binds to TTX and saxitoxin from plasma of the puffer fish *T. pardalis,* and its molecular weight was estimated to be about 104 kDa by SDS–PAGE. Furthermore, TTX-binding proteins are also applicable to affinity chromatography for TTX as ligands and the developed method is useful for purifying and analysing TTX in biological samples (Nagshima, Watanabe, Arita, Shimakura, & Shiomi, 1997; Shiomi et al., 1993).

In this study, we have tried to purify the particular proteins "TTX-binding high molecular weight substances (TTX-binding HMWS)" from the toxic gastropods *Polinices didamy*, *Natica lineata*, *O. miniacea*, *O. mustelina* and *O. hirasei*, and to characterise the TTX-binding protein from *N. lineata*.

2. Materials and method

2.1. Materials

Toxic gastropods *O. miniacea*, *O. mustelina* and *O. hirasei* were collected in Pingtung County, and *P. didamy* and *N. lineata* were collected in Chaiyi County. Furthermore *N. lineata* were collected for four seasons (November 2003 to December 2004). ICR strain mice were purchased from the National Animal Laboratory (Taiwan). Authentic TTX and paralytic shellfish poison (PSP), composed of gonyautoxin 1–4 (GTX1-4), were purified from puffer fish *Takifugu oblongus* liver and from the toxic alga *Alexandrium minutum*, respectively, as previously described (Hwang, Noguchi, Arakawa, Abe, & Hashimoto, 1988; Hwang et al., 1999).

The muscle of the gastropod (40 g) was homogenised with three volumes of 0.05 M NaCl in 0.01 M Tris–HCl (pH 7.4) for 5 min, and centrifuged (3000*g*, 20 min). The supernatant was concentrated with a Diaflo YM-2 membrane (Amicon, Beverly, MA, USA). The concentrate was regarded as the muscle extract of the toxic gastropod. Protease, DNase I, RNase T2, amyloglucosidase and α-amylase were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Gel filtration on Sepharose CL-6B

Column chromatography was performed to examine the TTX-binding capacity of the TTX-binding high molecular weight substances (HMWS). In order to determine the TTX-binding capacity of the TTX-binding HMWS, 10 ml (200 MU) of muscle extract were loaded onto a Sepharose CL-6B column (2.5 × 90 cm, Amersham Pharmacia Biotech, Little Chalfont, UK). The column was eluted with 0.05 M NaCl in 0.01 M Tris–HCl (pH 7.4) at a flow rate of 0.8 ml/min and fractions of 8 ml were collected. Each fraction was monitored by measuring its absorbance at 280 nm. The protein content was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard protein. Total neutral carbohydrate content was measured by the phenol–sulphuric acid method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956) using D-glucose as a standard.

2.3. Analysis of TTX-binding capacity

Each fraction from the Sepharose CL-6B column, after lyophilization and extraction with 1 ml of 0.03 M acetic acid, was analysed for TTX by assay of toxicity (Hwang & Jeng, 1991) and HPLC (Nagashima, Maruyama, Noguchi, & Hashimoto, 1987). The protein in the TTX-containing fraction was regarded as TTX-binding HMWS. TTX-binding HMWS fractions of *N. lineata* were combined, concentrated with a Diaflo YM-2 membrane desalted and chromatographed with a Q Fast-Flow anion exchanger.

2.4. Neutralisation tests

The TTX-binding HMWS (10 mg/ml) of *N. lineata* was mixed with TTX toxin solution (5 MU/ml) and PSP toxin solution (6.5 MU/ml) at a volume ratio of 1:1, respectively. After being kept at 4 °C for 5 min, the mixture was assayed for toxicity by mouse assay (AOAC, 1995; Hwang & Jeng, 1991). In control experiments, the TTX-binding HMWS was replaced by 10 mM sodium phosphate buffer (pH 7.0), containing bovine serum albumin at the same protein concentration as TTX-binding HMWS five replicates were performed for each assay.

2.5. Stability tests

The lyophilised solid (245 mg) of *N. lineata* TTX-binding HMWS was dissolved in 5 ml of 0.05 M NaCl in 0.01 M Tris–HCl (pH 7.4) buffer. Each 1 ml aliquot was incubated at 37 °C for 30 min either with 10 mg (80 U) of protease at pH 7.4, 2000 U of DNase I at pH 7.7, 500 U of RNase T2 at pH 8.3, amyloglucosidase or α-amylase. For hydrolysis with HCl, a 1 mL aliquot was mixed with an equal volume of 1 M HCl and heated in boiling water (100 °C) for 10 min. Hydrolysates were neutralised and diluted to 5 ml with 0.03 M acetic acid. Each 1 ml of diluted hydrolysate was injected into mice for toxicity assay (5 times per hydrolysate).

2.6. Purification of TTX-binding protein

TTX-binding HMWS of *N. lineata* was desalted before applying to a Q Fast-Flow anion exchanger (5 mL, Amersham Pharmacia Biotech). TTX-binding HMWS of *N. lineata* was applied to Q Fast-Flow anion exchanger equilibrated with 0.05 M NaCl in 0.01 M Tris–HCl (pH 7.4) starting buffer, and then eluted with 0.5 M NaCl in starter buffer (elution buffer) at a flow rate 0.5 ml/min. Fractions of 1 ml were collected. Each fraction was determined for protein content and TTX-binding capacity. The protein which had TTX-binding capacity was regarded as the TTX-binding

protein. TTX-binding protein of *N. lineata* was finally purified by repeated gel filtration chromatography on a BioSep-SEC-S 2000 column (300 × 7.8 mm, Phenomenex, Torrance, CA, USA), which was eluted with 0.16 M NaCl in 0.01 M Tris–HCl (pH 7.4) at a flow rate of 0.8 ml/min. TTX-binding HMWS was monitored by absorbance at 280 nm. The fractions with TTX-binding capacity were eluted near the void volume of the column after desalting. All procedures for the purification of the TTX-binding HMWS were conducted at about 4 °C.

2.7. Electrophoretic analyses

The purified TTX-binding protein of *N. lineata* was analysed by native polyacrylamide gel electrophoresis (PAGE) and SDS–PAGE. In all the PAGEs, the gels were stained with Coomassie Brilliant Blue R-250. Native-PAGE was carried out on a Phastgel homogeneous 7.5 with a Phastgel native buffer strip. SDS–PAGE was carried out on a Phastgel homogeneous 7.5 with Phastgel SDS buffer strip. Prior to loading, the sample was dissolved in 0.01 M Tris–HCl buffer (pH 7.4) containing 1% SDS or the buffer containing 1% SDS and 2.5% β-mercaptoethanol, and heated in a boiling water for 5 min. Native and SDS high molecular weight calibration kits (Amersham Pharmacia Biotech) were used as references.

3. Results and discussion

Fig. 1 illustrates the gel filtration pattern on Sepharose CL-6B of the muscle extract from the toxic gastropod *N. lineata*. Four main peaks were observed when monitored by absorbance at 280 nm. TTX was detected in two fraction groups, fractions 28–33 and 78–84. It implied that the former fraction group contained TTX-binding HMWS (Fraction II), while the latter consisted of TTX in its free form.

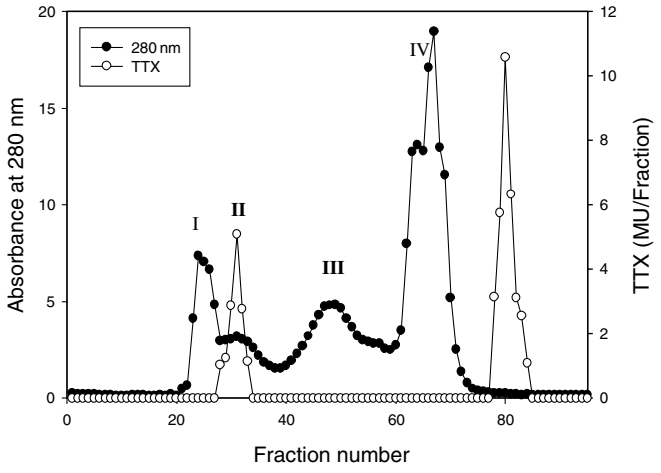

Fig. 1. Gel filtration chromatograms on a Sepharose CL-6B column of *Natica lineata* muscle extract.

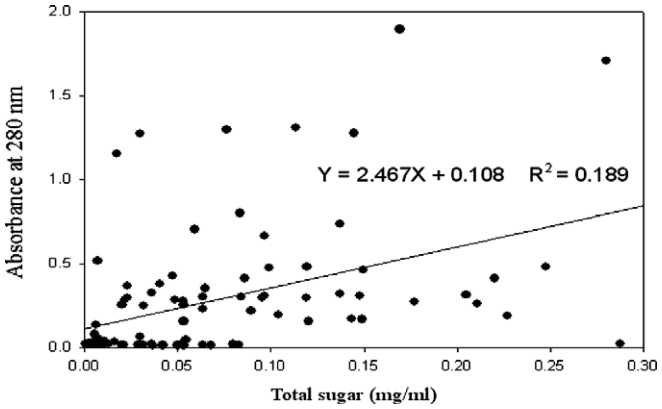

Fig. 2. The correlation between amount of total sugar and A280 of each fraction after chromatographing on Sepharose CL-6B column of muscle extract of *Natica lineata*.

A 362 mg sample of *N. lineata* TTX-binding HMWS had a TTX-binding capacity of about 224 MU (specific activity = 0.62 MU/mg). The TTX-binding capacities of the TTX-binding HMWS from *P. didamy*, *O. miniacea*, *O. mustelina* and *O. hirasei* were 0.12, 0.45, 0.28 and 0.35 MU/mg protein, respectively. It was found that the yield from *N. lineata* was constant throughout the whole year and the seasonal variation of toxin-binding capacity was small. There was almost no correlation between the amount of total sugar and A_{280} of each fraction of Sepharose CL-6B (Fig. 2), and the TTX-binding HMWS could not bind to a Con-A column either, which indicated that HMWS was not a glycoprotein.

In the neutralisation tests where TTX/PSP toxin solution mixed with the TTX-binding HMWS of *N. lineata* was injected intraperitoneally into mice, the mice all died. Therefore, the TTX-binding HMWS of *N. lineata* showed no significant neutralising effect on the lethal activity of TTX/PSP.

After injecting the protease and HCl hydrolysate into five mice, all mice showed symptoms characteristic of TTX poisoning and died. On the other hand, none of the DNase I, RNase T2, amyloglucosidase and α-amylase hydrolysates showed any neurotoxic signs in mice (Table 1). The TTX-binding HMWS of *N. lineata* was chromatographed on a Q Fast-Flow anion exchanger column, and

Table 1
Toxicity of TTX-binding high molecular weight substance (HMWS) of *Natica lineata* after being hydrolysed with enzyme and HCl

Treatment of TTX-binding HMWS	No. dead/No. examined	Toxicity (MU/ml)
Protease	5/5	4.39
HCl	5/5	4.72
HCl + heating	5/5	4.86
Heating	5/5	3.90
RNase T2	0/5	ND
DNase I	0/5	ND
Amyloglucosidase	0/5	ND
α-Amylase	0/5	ND

the peak of TTX-binding protein was eluted at about 0.16 M NaCl (Frs. 16–18) from the Q Fast-Flow anion exchanger (Fig. 3). The amount of TTX-binding protein in *N. lineata* was 13 mg and its TTX-binding capacity was 46 MU. Hence, the specific activity of crude TTX-binding protein was 3.5 MU/mg. Finally, the protein having the TTX-binding capacity eluted as a single peak on a BioSep-SEC-S 2000 column (Fig. 4), and 11 mg of the protein had a TTX-binding capacity of about 47 MU (specific activity = 4.2 MU/mg).

The purified TTX-binding protein gave only one band on native-PAGE, confirming its homogeneity, and the molecular weight estimated by native-PAGE, was about 434 kDa (Fig. 5). On the other hand, the molecular weights estimated by SDS–PAGE were about 272 kDa and 47 kDa under reducing conditions (Fig. 6). These results indicated that the TTX-binding protein comprised at least two kinds of subunit.

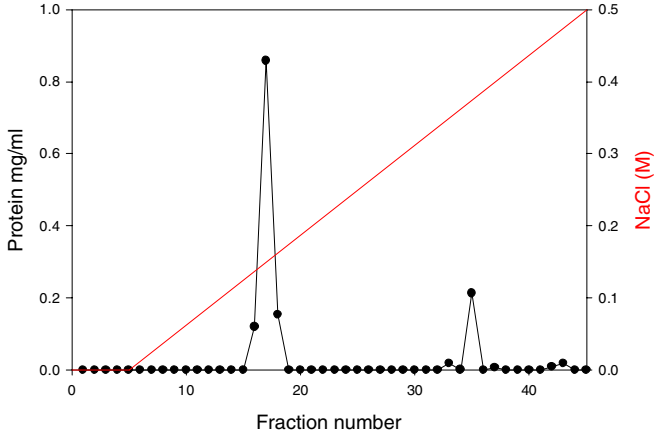

Fig. 3. Ion exchange chromatograms on a Q Fast-Flow anion column of TTX-binding protein of *Natica lineata*.

Shore crab *H. sanguineus* (non-toxic) contained a soluble protein that could bind TTX and its molecular weight was estimated to be about 400 kDa. It was comprised of two subunits with molecular weights of about 72 kDa and 82 kDa (Nagashima et al., 2002). The soluble protein of *H. sanguineus* could neutralise the toxicity of TTX, so it was thought to possess a defence function against TTX for *H. sanguineus* (Nagashima et al., 1987). A survey of TTX-binding activity in the plasmas of toxic puffer fish and non-toxic fish revealed that the toxic puffer fish possessed high TTX-binding activity and non-toxic fish did not. A TTX-binding protein has been purified from the plasma of the toxic puffer fish *Takifugu niphobles*, and its molecular weight was estimated to be about 116 kDa by SDS–PAGE. This binding ability suggested that the TTX-binding protein takes part in TTX transfer and transport in the puffer fish (Matsui et al., 2000). Furthermore, a novel soluble glycoprotein that binds to TTX and STX was isolated from the plasma of the toxic puffer fish *Fugu pardalis*, and its molecular weight was estimated to be about 104 kDa by SDS–PAGE (Yamashita-Yotsu et al., 2001).

This study revealed the occurrence of a TTX-binding protein with a molecular weight of about 434 kDa in the muscle of the toxic gastropod *N. lineata*, which corresponds to the TTX-binding protein of about 400 kDa in the haemolymph of the shore crab *H. sanguineus*

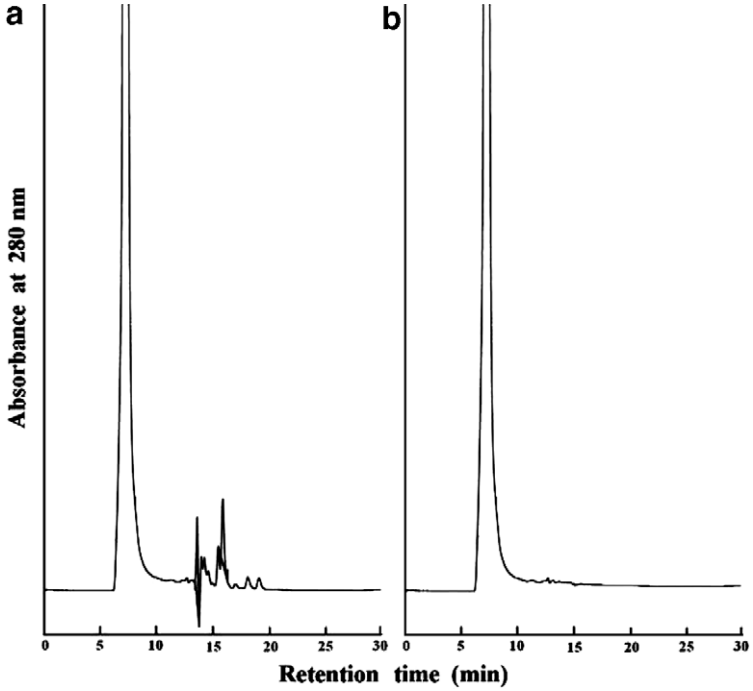

Fig. 4. HPLC of TTX-binding protein of *Natica lineata* on a BioSep-SEC-S 2000 column. (a) Active fraction by Q Fast-Flow anion exchanger. (b) Active fraction by Q Fast-Flow anion exchanger chromatographed two times.

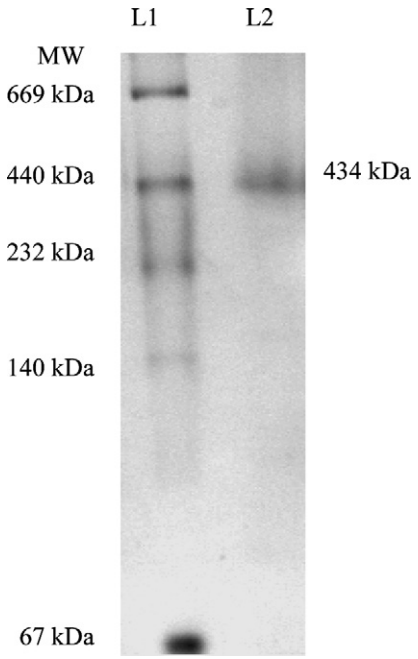

Fig. 5. Native-PAGE of *Natica lineata* TTX-binding protein. Lane 1, protein standard; lane 2, *Natica lineata* TTX-binding protein.

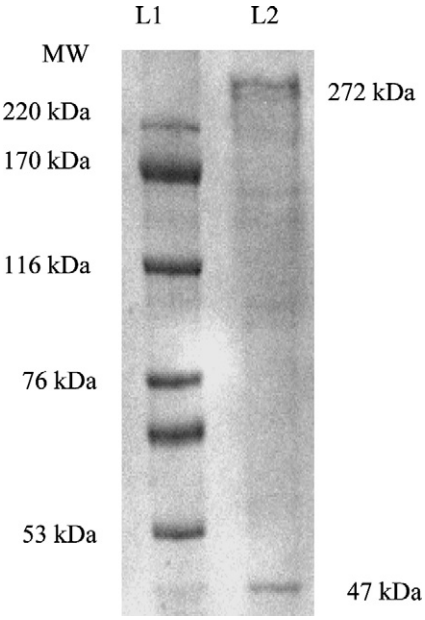

Fig. 6. SDS–PAGE of *Natica lineata* TTX-binding protein. Lane 1, protein standard; lane 2, *Natica lineata* TTX-binding protein.

(Nagashima et al., 2002). The TTX-binding protein was successfully purified by them to obtain a homogeneous substance by Sepharose CL-6B chromatography, affinity chromatography on Q Fast-Flow anion exchanger and HPLC on BioSep-SEC-S 2000 column. The protein comprised at least two subunits with molecular weights of about 272 kDa and 47 kDa, strongly suggesting that the TTX-binding protein is a polymeric form.

The purified TTX-binding protein had a specific TTX-binding capacity of 4.8 MU/mg against the lethal effect of TTX. But extra TTX added into the TTX-binding HMWS showing no neutralising ability. This fact indicated that TTX-binding protein in the toxic gastropod *N. lineata* may take part in TTX transfer and transport. The TTX-binding protein in *H. sanguineus* has different properties from *N. lineata*. That form is highly resistant to TTX, although it contains no detectable amount of TTX, and its TTX-binding protein was examined for neutralising effects against the lethal activity of TTX (Shiomi, Yamaguchi, Kikuchi, Yamamori, & Matsui, 1992).

Several TTX and/or PSP-binding proteins have so far been detected in plasma or haemolymph in animals: saxiphilin from the plasma of the bull frog (Li & Moczydlowski, 1991), TTX-neutralising factors from horseshoe crab haemolymph (Yeo, Ding, & Ho, 1996), saxitoxin-binding proteins from the plasma or haemolyph of reptiles or arthropods (Llewellyn, 1997; Llewellyn, Bell, & Mocyzdlowski, 1997), and TTX-binding protein from puffer fish plasma (Matsui et al., 2000). Among these TTX and/or PSP-binding proteins, saxiphilin and the saxitoxin-binding proteins can bind to saxitoxin only, not to TTX. We are studying the TTX-binding proteins in the gastropod *N. lineata* further to elucidate its ability in binding PSP.

4. Conclusion

A TTX-binding high molecular weight substance was found in the muscle of five toxic gastropods. This substance was identified as a protein. The molecular weight of the TTX-binding protein of *N. lineata* was estimated to be about 434 kDa. It comprised two subunits with molecular weights of about 272 kDa and 47 kDa, respectively, using SDS–PAGE.

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References

AOAC (1995). *Official methods of analysis of AOAC international* (16th ed.). Arlington, VA: AOAC International.

Dubois, M., Gilles, A. K., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetry method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350–356.

Hwang, D. F., & Jeng, S. S. (1991). Bioassay of tetrodotoxin by using ICR strain male mouse. *Journal of Chinese Biochemical Society, 20*, 80–86.

Hwang, D. F., Noguchi, T., Arakawa, O., Abe, T., & Hashimoto, K. (1988). Toxicological studies on several species of puffer in Taiwan. *Nippon Suisan Gakkaishi, 54*, 2001–2008.

Hwang, D. F., Tsai, Y. H., Liao, H. J., Matsuoka, K., Noguchi, T., & Jeng, S. S. (1999). Toxins of the dinoflagellate *Alexandrium minutum* Halim from the coastal waters and aquaculture ponds in southern Taiwan. *Fishery Science, 65*, 171–172.

Li, Y., & Moczydlowski, E. G. (1991). Purification and partial sequencing of saxiphilin, a saxitoxin-binding protein from the bull frog, reveals homology to transferring. *Journal of Biological Chemistry, 266*, 15481–15487.

Llewellyn, L. E. (1997). Haemolymph protein in xanthid crabs: its selective binding of saxitoxin binding site of the Na^+ channel. *Biophysical Journal, 66*, 1–13.

Llewellyn, L. E., Bell, P. M., & Mocyzdlowski, E. G. (1997). Phylogenetic survey of soluble saxitoxin-binding activity in pursuit of the function and molecular evolution of saxiphilin a relative of transferring. *Proceedings of the Royal Society London, 264*, 891–902.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Mahmud, Y., Okada, K., Takatani, T., Kawatsu, K., Hamano, Y., Arakawa, O., et al. (2003). Intra-tissue distribution of tetrodotoxin in two marine puffers *Takifugu vermicularis* and *Chelonodon patoca*. *Toxicon, 41*, 13–18.

Matsui, T., Yamamori, K., Furukawa, K., & Kono, M. (2000). Purification and some properties of a tetrodotoxin binding protein from the blood plasma of kusafugu, *Takifugu niphobles*. *Toxicon, 38*, 463–468.

Nagashima, Y., Maruyama, J., Noguchi, T., & Hashimoto, K. (1987). Analysis of paralytic shellfish poison and tetrodotoxin by ion-pairing high performance liquid chromatography. *Nippon Suisan Gakkaishi, 50*, 85–88.

Nagshima, Y., Watanabe, K., Arita, J., Shimakura, K., & Shiomi, K. (1997). Affinity chromatography using tetrodotoxin-binding high molecular weight substances in the shore crab *Hemigrapsus sanguineus* as ligands: usefulness in analyzing tetrodotoxin in biological samples. *Journal of Natural Toxins, 6*, 293–306.

Nagashima, Y., Ohgoe, H., Yamamoto, K., Shimakura, K., & Shiomi, K. (1998). Resistance of non-toxic crabs to paralytic shellfish poisoning toxin. In B. Reguera, J. Banco, M. L. Fernandez, & T. Wyatta (Eds.), *Harmful algae* (pp. 604–606). Santigago de Compostela: Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO.

Nagashima, Y., Yamamoto, K., Shimakura, K., & Shiomi, K. (2002). A tetrodotoxin-binding protein in the hemolymph of shore crab *Hemigrapsus sanguineus*: purification and properties. *Toxicon, 40*, 753–760.

Shiomi, K., Yamaguchi, S., Kikuchi, T., Yamamori, K., & Matsui, T. (1992). Occurrence of tetrodotoxin-binding high molecular weight substances in the body fluid of shore crab (*Hemigrapsus sanguineus*). *Toxicon, 30*, 1529–1537.

Shiomi, K., Yamaguchi, S., Shimakura, K., Nagashima, Y., Yamamori, K., & Matsui, T. (1993). Affinity chromatographic purification of tetrodotoxin by use of tetrodotoxin-binding high molecular weight substances in the body fluid of shore crab (*Hemigrapsus sanguineus*) as ligand. *Toxicon, 31*, 1615–1618.

Yamashita-Yotsu, M., Sugimoto, A., Terakawa, T., Shoji, Y., Miyazawa, T., & Yasumoto, T. (2001). Purification, characterization, and cDNA cloning of a novel soluble saxitoxin and tetrodotoxin binding protein from plasma of the puffer fish, *Fugu pardalis*. *European Journal of Biochemistry, 268*, 5937–5946.

Yeo, D. S. Y., Ding, J. L., & Ho, B. (1996). Neuroblastoma cell culture assay shows that *Carcinoscorpius rotundicauda* haemolymph neutralizes tetrodotoxin. *Toxicon, 34*, 1054–1057.