

Accumulation and depuration of paralytic shellfish poisoning toxins by laboratory cultured purple clam *Hiatula diphos* Linnaeus

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Abstract

Purple clams (*Hiatula diphos* Linnaeus) accumulate paralytic shellfish poisoning (PSP) toxins produced by a toxic strain of the dinoflagellate *Alexandrium minutum* Halim in a laboratory study. The maximal toxicity of PSP toxins attained 31.3 MU/g after 20 days exposure. The toxin profile of *H. diphos* was similar to that reported for *A. minutum* at the end of the exposure period; and GTX1 was dominant. GTX congeners were found in muscle on day 16 and day 20, these substances could be detected during the depuration period as well. GTX1 was detected in the siphon only on day 32. The results show that *H. diphos* accumulates PSP toxins according to the amount and toxin profile of ingested *A. minutum*.

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1. Introduction

Paralytic shellfish poisoning (PSP) toxins are potent marine neurotoxins, which block sodium channels of the neuronal cell membrane (Kao, 1993). They are produced by dinoflagellates such as *Alexandrium*, *Pyrodinium*, and *Gymnodinium*, and to date more than 20 toxins including the analogues gonyautoxin (GTX1–4) have been reported (Andrinolo et al., 2002). Toxic algae of the genus *Alexandrium* are the dominant sources of PSP toxins in contaminated bivalves (Li and Wang, 2001).

In Taiwan, *Alexandrium minutum* is a species of dinoflagellate producing PSP toxins (Hwang et al., 1999). The purple clam *Hiatula rostrata* Lightfoot is known to retain and accumulate high PSP toxin levels during natural *A. minutum* blooming events (Hwang et al., 1987, 1999;

Chen, 1998). GTX1, 2, 3 and 4 are the major toxins that occur in *A. minutum* and in the purple clam *H. rostrata* (Hwang et al., 1999). We previously reported (Chen and Chou, 2001a,b) accumulation of PSP toxins in *H. rostrata* from laboratory cultured *A. minutum*. In these studies, we found clear differences in the toxin profiles of the digestive gland, muscle and siphon.

Long term monitoring of PSP in Taiwan has found most of the toxic incidents to be related to *H. rostrata*, whereas no toxicity cases related to consumption of the purple clam *H. diphos* Linnaeus have been reported (Chen, 1998). Furthermore, data on toxin accumulation and depuration of *H. diphos* are lacking. The main purpose of this study was to determine the extent to which PSP toxins could accumulate in the purple clam *H. diphos* during a continuous toxic algae feeding regime, and to define the time course for PSP toxin accumulation and depuration. Thereafter, our reasons would allow a direct comparison to be made with data previously reported for *H. rostrata* (Chen and Chou, 2001a).

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2. Materials and methods

2.1. Organisms

Toxic strain AMTK-4 of *A. minutum* Halim was isolated from the Tungkang area where PSP occurred due to the ingestion of purple clams *H. rostrata*. The unialgal isolates were batch cultured and illuminated with continuous light of $60 \mu\text{E m}^{-2} \text{s}^{-1}$ at 20–22. Nontoxic algae *Pavlova salina* cultures, used as feed for depuration period, were also maintained under the same conditions. Algal cells of both toxic and nontoxic species were harvested for feeding experiments during the exponential growth phase.

Purple clams (*Hiatala diphos* Linnaeus), size range 6–8 cm, were collected from an aquaculture pond at Shian-shi, Chang-Hua Prefecture.

2.2. Culture environment

Ninety purple clams were brought to the laboratory and placed in a 130 L tank with filtered sea water (25 ppt salinity). Clams were maintained at $23 \pm 2^\circ\text{C}$ with continuous aeration throughout the holding period. During the exposure period, lasting 24 days, the clams were fed with *A. minutum* (AMTK-4) at 1-day interval and exposed approximately to 2.97×10^8 cells. During the depuration period, starting at day 25 and lasting 24 days, clams were fed with *P. salina* at 3-day intervals and exposed approximately to 1.54×10^8 cells.

2.3. HPLC of PSP toxins

The toxicity and toxin profile of purple clams were monitored during the contamination and depuration periods. The purple clams ($n=4$) were periodically (day 4, day 8, day 12, day 16, day 20, day 24, day 28, day 32, day 36, day 40, day 44, and day 48, respectively) removed for toxin analysis after dissection into three parts: digestive gland, siphon and muscle (including the foot, gill, adductor and mantle). The method of extraction of PSP toxins was according to Chen and Chou (1998). Tissues were homogenized with 15 ml of 0.1 M HCl, the extracts were defatted twice with chloroform, and the aqueous layer was ultrafiltered by centrifugation (10,000 MW cut-off). Subsequently, $10 \mu\text{l}$ of the supernatant were injected into the HPLC with a post-column reaction system (Chen and Chou, 2002a). The column used was a $5 \mu\text{m}$, $250 \times 4.6 \text{ mm}$ Cosmosil 5C18-AR column at a flow rate of 0.8 ml/min. Fluorescent PSP toxin derivatives were detected using a Hitachi F-1000 spectrophotometer with excitation at 336 nm and emission at 390 nm.

2.4. Calculation of toxicity from HPLC data

The conversion of peak areas of HPLC-resolved toxins into mouse units was according to the following

formula: Toxicity (MU/ml) = $(19.7A_1 + 0.57A_2 + 1.61A_3 + 8.11A_4) \times 10^{-6}$, where MU is 1 mouse unit and A_1 , A_2 , A_3 , and A_4 represent the peak areas of the resolved toxins, GTX1, 2, 3, and 4, respectively. The suitability of the formula was based on the fact that there were no resolved peaks other than those corresponding to the above four toxins in the tested material. The calibration factors for each toxin were adopted from mouse assays of partially purified toxin mixtures with the reported specific toxicity (Genenah and Shimizu, 1981) and the reported specific fluorescent response (Sullivan et al., 1985). This provides a way to convert the HPLC data to the generally accepted mouse toxicity units as STX equivalents (AOAC, 1995). In our case it was observed that 1 MU (ICR strain, male, 20 g) was equivalent to $0.259 \mu\text{g/ml}$ STX by the calibration procedure suggested by the AOAC (Chou, 1999). According to the above formula and the corresponding mouse responses to each toxin, the specific amount (μmol) of each individual toxin could thus be calculated from its peak area. Correlation factors for each toxin were 1.2×10^{-10} , 7.2×10^{-12} , 7.2×10^{-12} , and 1.2×10^{-10} for GTX-1, 2, 3, and 4, respectively.

3. Results

In this experiment, the toxicity of *A. minutum* was $7.12 \text{ MU}/10^6$ cell. In the purple clams *H. diphos*, the maximal toxicity levels were reached on day 20 of exposure period ($8.1 \mu\text{g}$ STX eq (31.3 MU)/g shellfish meat) (Fig. 1). On day 24, the accumulated toxicity was no more increasing although the purple clams ingested more toxic algae. During depuration period, the clams lost 50% of their body burden of toxin on day 28 (Fig. 1). Subsequently, the toxicity increased on day 36 and day 40, we suggested that the purple

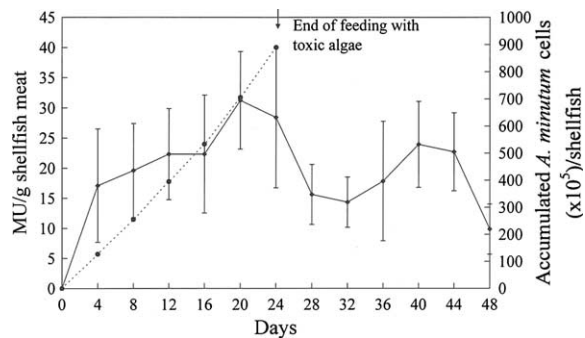


Fig. 1. The amount of ingested toxic dinoflagellate (*Alexandrium minutum*) cells (dashed line) and the mouse unit toxicity detected in purple clams *H. diphos* during the accumulation and depuration period (solid line). Algal cells taken by each of the purple clams were calculated from the reduction of the cells. Values of toxicity are means \pm SE converted from HPLC analysis of four different clams.

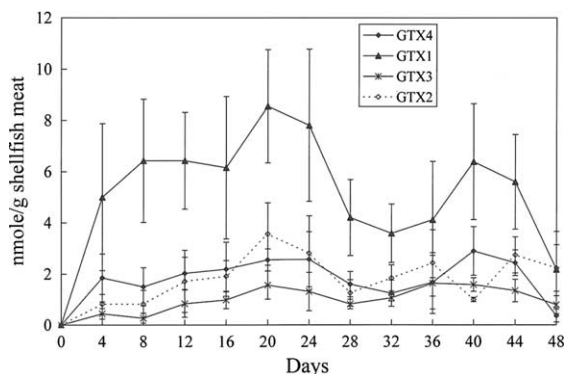


Fig. 2. Changes of PSP toxin compositions in purple clams *H. diphos*, during the feeding of *A. minutum* and following feeding of nontoxic algae. Values of toxin amount are means \pm SE converted from HPLC analysis of four different clams.

clams had ingested toxic algal cells from cysts in the sediment.

During the exposure period, GTX1 was the major toxin in the shellfish, and the toxin profile was similar to that of *A. minutum* (Fig. 2). GTX4 and GTX1 accounted for more than 85% of the molar toxin content in the shellfish. During the depuration period, GTX1 was the major toxin as well.

Furthermore, purple clams *H. diphos* concentrated most of the algal toxins in the digestive glands. During the accumulation and depuration period, GTX1 was the major toxin in the digestive gland (Fig. 3). In non-visceral tissues, the muscle started to accumulate GTX congeners on day 16 and day 20, and these toxins could be detected during the depuration period as well (Fig. 4). That is, some of PSP toxins were translocated from digestive gland to muscle. In muscle, the values of means \pm SE are huge so that there is unlikely to be a difference between any of these GTX congeners. It is suggested that toxin transformation might occur in muscle. However, the siphon became toxic only on

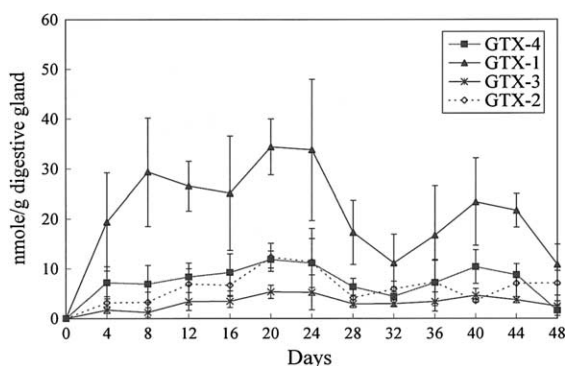


Fig. 3. PSP toxin compositions in digestive glands of purple clams *H. diphos*, during the feeding of *A. minutum* and following feeding of nontoxic algae. Values of toxin amount are means \pm SE converted from HPLC analysis of four different clams.

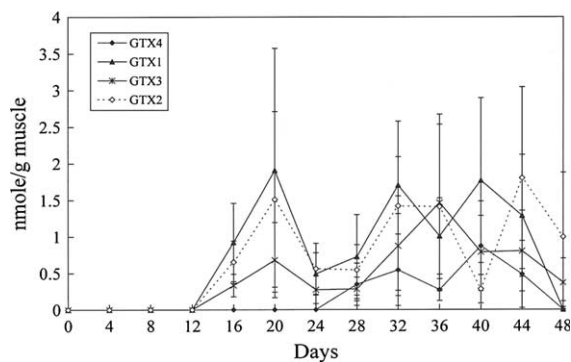


Fig. 4. Changes of PSP toxin compositions in muscles of purple clams *H. diphos*, during the feeding of *A. minutum* and following feeding of nontoxic algae. Values of toxin amount are means \pm SE converted from HPLC analysis of four different clams.

day 32, the toxin could be detected was GTX1. We suggest that the siphon tended to selectively retain GTX1.

4. Discussion

From the above results, we suggest that purple clams would undergo toxin accumulation within a short time during exposure to high concentrations of *A. minutum*. The maximal toxicity observed in another species of purple clam *H. rostrata* is 40.6 MU/g (Chen and Chou, 2001b), which is higher than *H. diphos*. It is noteworthy that *H. diphos* attains the maximal toxin level on day 20 and the toxicity is not increasing on day 24. We suggest once that purple clams reach the feeding capacity of toxic algae, most of the algae become pseudofaeces and are unable to enter the digestive gland. This might be the reason why the toxicity decreased even on day 24 in *H. diphos* when it is ingesting more toxic *A. minutum*. Furthermore, the toxicity increased on day 36 and day 40. *H. diphos* could ingest toxic cysts from sediment. In general, the toxicity of cyst is higher than algal cell (Oshima et al., 1992).

We found that *H. diphos* showed toxicity in muscle earlier than other species of purple clam *H. rostrata* (Chen and Chou, 2001a). It is suggested that the half-life of PSP toxins in *H. rostrata* is shorter than in *H. diphos*.

Our results showed that siphon was toxic during depuration period. In previous reports deal with other species of purple clam *H. rostrata*, shows similar results (Chen and Chou, 2001a, 2002b). Furthermore, GTX1 was only detectable in siphon on day 32, which suggests that the half-life of PSP toxins in siphon was short. However, butter clam, *Saxidomus giganteus* accumulates saxitoxin in the siphon and elimination from it is slow (Smolowitz and Doucette, 1995).

In Taiwan, purple clam *H. rostrata* is the only shellfish species reported to result in poisoning incidents (Hwang et al., 1999; Chen, 1998). The number of toxic algae was

very low or even not detected in the habitat of *H. diphos* (Chen and Chou, 2002b). Despite the fact that no wild *H. diphos* has so far been found to be toxic in the natural environment, our experiments clearly show that this species is capable of accumulating PSP toxins after feeding with toxic *A. minutum*. Comparing the results of *H. diphos* with our previous reports of *H. rostrata* (Chen and Chou, 2001a), there is no significant difference in the profile of toxin accumulation and depuration. The salinity which wild *H. diphos* inhabits is higher than *H. rostrata* culture ponds. Low salinity stimulated the algal cells to produce high amount of GTX1, which was the major toxin during contamination period (Hwang and Lu, 2000). Furthermore, intensive culture of *H. rostrata* rather than *H. diphos* is common in southwestern Taiwan. We suggest that the geographic distribution of toxic purple clams were owing to the different environment and culture behavior that brought the blooming of toxic algae and contaminated the clams.

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References

- Andrinolo, D., Iglesias, V., Garcia, C., Lagos, N., 2002. Toxicokinetics and toxicodynamics of gonyautoxins after an oral toxin dose in cats. *Toxicon* 40, 699–709.
- AOAC (Association of Official Analytical Chemist), 1995. Paralytic shellfish poisons biological method final action. In: Horwitz, W. (Ed.), *Official Methods of the Association of Official Analytical Chemists*, 15th ed. AOAC, Washington, DC, pp. 21–22.
- Chen C.Y., 1998. Purple clam accumulation. *Food Chain Transmission and Ichthyotoxicity Studies of Paralytic Shellfish Poisons from Alexandrium minutum* Halim. Ph.D. thesis, National Taiwan University, Taipei, p. 20.
- Chen, C.Y., Chou, H.N., 1998. Transmission of the paralytic shellfish poisoning toxins, from dinoflagellate to gastropod. *Toxicon* 36, 515–522.
- Chen, C.Y., Chou, H.N., 2001a. Accumulation and depuration of paralytic shellfish poisoning toxins by purple clam *Hiatula rostrata* Lightfoot. *Toxicon* 39, 1029–1034.
- Chen, C.Y., Chou, H.N., 2001b. Paralytic shellfish poisoning toxins accumulation in purple clam *Hiatula rostrata* and toxic effect on milkfish *Chanos chanos* larval fish. *J. Nat. Toxins* 10, 299–305.
- Chen, C.Y., Chou, H.N., 2002a. A modified HPLC method for analysis PSP toxins in dinoflagellate *Alexandrium minutum*. *Food Res. Int.* 35, 715–720.
- Chen, C.Y., Chou, H.N., 2002b. Fate of paralytic shellfish poisoning toxins in purple clam *Hiatula rostrata*, in outdoor culture and laboratory culture. *Mar. Pollut. Bull.* 44, 733–738.
- Chou, H.N., 1999. Paralytic shellfish poisoning toxins. In: Chou, H.N. (Ed.), *Manual on Microalgal Toxin Detection in Seafood Products*. DOH, Taipei, pp. 23–44.
- Genenah, A.A., Shimizu, Y., 1981. Specific toxicity of paralytic shellfish poisons. *J. Agri. Food Chem.* 29, 1289–1291.
- Hwang, D.F., Noguchi, T., Nagashima, Y., Liao, I.C., Hashimoto, K., 1987. Occurrence of paralytic shellfish poison in the purple clam *Soletellina diphos* (Bivalve). *Nippon Suisan Gakkaishi* 53, 623–626.
- Hwang, D.F., Lu, Y.H., 2000. Influence of environmental and nutritional factors on growth, toxicity, and toxin profile of dinoflagellate *Alexandrium minutum*. *Toxicon* 38, 1491–1503.
- 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. *Fish Sci.* 65, 171–172.
- Kao, C.Y., 1993. Paralytic shellfish poisoning. In: Falconer, I.R. (Ed.), *Algal Toxins in Seafood and Drinking Water*. Academic Press, London and New York, pp. 75–86 (chapter 4).
- Li, S.C., Wang, W.X., 2001. Radiotracer studies on the feeding of two marine bivalves on the toxic and nontoxic dinoflagellate *Alexandrium tamarense*. *J. Exp. Mar. Biol. Ecol.* 263, 65–75.
- Oshima, Y., Bolch, C.J., Hallegraef, G.M., 1992. Toxin composition of resting cysts of *Alexandrium tamarense* (Dinophyceae). *Toxicon* 30, 1539–1544.
- Smolowitz, R., Doucette, G., 1995. Immunohistochemical localization of saxitoxin in the siphon epithelium of the butter clam, *Saxidomus giganteus*. *Biol. Bull.* 189, 229–230.
- Sullivan, J.J., Wekell, M.M., Kentala, L.L., 1985. Application of HPLC for the determination of PSP toxins in shellfish. *J. Food Sci.* 50, 26–29.