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Fate of paralytic shellfish poisoning toxins in purple clam *Hiatula rostrata*, in outdoor culture and laboratory culture

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Abstract

Purple clams (*Hiatula rostrata* Lighttoot) accumulate paralytic shellfish poisoning (PSP) toxins produced by a toxic strain of the dinoflagellate *Alexandrium minutum* Halim. The results confirm the data of our previous study concerning the muscle and siphon that were not showing a gradual rise in toxicity when shellfish accumulated more *A. minutum*. However, muscle and siphon are intermittently toxic both in exposure and depuration period in laboratory cultured purple clams. PSP toxins were detected in outdoor cultured purple clams, whereas no *A. minutum* were found in the culture pond during most of the survey time. The outdoor cultured purple clams need longer time to decrease toxicity to allowable levels than laboratory cultured purple clams. It was shown that laboratory data may not predict times over which pond-cultured purple clams may prove toxic to consumers.
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1. Introduction

We previously reported (Chen and Chou, 2001) accumulation in purple clams (*Hiatula rostrata*) of paralytic shellfish poisoning (PSP) toxins from toxic microalgae of *Alexandrium minutum*. There were rather marked differences in the comparative toxin profiles of digestive gland, muscle and siphon. In digestive gland, GTX1 was the major PSP toxin. In muscle and siphon, GTX4 was the major toxin. The digestive gland was demonstrated to have the highest toxicity, followed by muscle and siphon. However, muscle and siphon only showed their toxicity after stop feeding with *A. minutum*, which is different from other bivalve species studied under similar experimental conditions (Bricelj et al., 1990, 1991). The muscle and siphon of many bivalve species showed toxicity both in the toxin accumulation and depuration period (Martin et al., 1990; Shumway et al., 1995). In outdoor culture pond, purple clams could accumulate PSP toxins to a fairly high toxicity and are capable of prolonged retention of PSP toxins (Hwang et al., 1987, 1995). Actually, purple clams are eaten as a whole, toxic specimens will badly impact on food safety. The major purpose of this paper is to explore the time when muscle and siphon will become toxic after feeding of toxic algae and toxin profile during exposure and depuration period in purple clams. Furthermore, compare the toxicity and toxin profile of outdoor and laboratory cultured purple clams.

2. Materials and methods

2.1. Materials and culture environment

Strain AMTK-1 of *A. minutum* Halim was isolated in 1986 from the Tungkang area where PSP occurred due to the ingestion of purple clams. The unialgal isolates were batch cultured in K-medium (Keller and Guillard, 1985) and illuminated with continuous light of 60 $\mu E\,m^{-2}\,s^{-1}$ at 20–22 °C. *Pavlova salina* cultures, used as nontoxic live feed for control purposes, were also maintained under the same conditions. Algal cells of both toxic and nontoxic species were harvested for feeding experiments in exponential growth phase.

Laboratory cultured purple clams (*H. rostrata* Lighttoot) average 6.86 cm (SE = 0.57, $n = 100$) in shell length

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(mean tissue wet weight = 8.58 g, SE = 1.04), were collected from an aquaculture pond in Tungkang, Pingtung Prefecture. The clams were brought to the laboratory and placed in a 130 l tank with filtered sea water (20 ppt salinity). Continuous aeration was carried out at 17 ± 2 °C throughout the holding period. An acclimation period of one week preceded the experiment in order to eliminate individuals that were too weak or showed physiological defects. During the exposure period, lasting 35 days, the clams were fed with *A. minutum* at 2-day intervals and exposed each time to $5.50 \pm 0.55 \times 10^8$ cells. During the depuration period, starting at day 36 and lasting 25 days, clams were fed with *P. salina* at 3-day intervals and exposed to $9.90 \pm 0.55 \times 10^{10}$ cells.

There were several consumers exerted PSP symptoms after eating outdoor cultured purple clams on June 18, 1996 in Tungkang, Pingtung Prefecture. We collected outdoor cultured purple clams intensively on June and then monthly from three close neighbor culture ponds (A, B, and C) in Tungkang, Pingtung Prefecture from June 1996 to January 1997 to analyze their toxicity and toxin profiles. The purple clams were kept alive and immediately transported to the laboratory. The specimens were examined for toxin composition as described below. The number of *A. minutum* in outdoor purple clam culture pond was monitored twice a month from January 1996 to January 1997. For qualitative sampling, a 10 μm net was hauled vertically from a depth of 2 m. Samples were preserved with Lugol's iodine and enumerated with microscope.

2.2. Assay of toxicity and HPLC

The toxicity and toxin profile of laboratory cultured purple clams were monitored during the exposure and depuration periods. The clams ($n = 4$) were periodically removed for toxin analysis after dissection into three parts: digestive gland, siphon and other tissues (including the foot, gill, adductor muscle and mantle). The method of extraction of PSP toxins was according to Chen and Chou (1998). Tissues were homogenized with 15 ml 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 μl of the supernatant were injected into the HPLC with a post-column reaction system (modified from Nagashima et al., 1987). The column used was a 5 μm, 250×4.6 mm^2 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.

2.3. Calculation

The conversion of peak areas of HPLC-resolved toxins into mouse units was according to the following formulation: Toxicity (MU/ml) = $(19.7A_1 + 0.57A_2 + 1.61A_3 + 8.11A_4) \times 10^{-6}$, where MU = 1 mouse unit and A_1, A_2, A_3, A_4 represented the peak areas of the resolved toxins, GTX-1, 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 μg/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

The average toxicities accumulated in each clam were indicated by the total mouse units that were converted from HPLC analysis of the shellfish extracts and the accumulated cells of toxic dinoflagellates taken by filter-feeding activities (Fig. 1). In *A. minutum*, the relative amount of individual toxins was constant throughout the feeding period. In this experiment, the toxicity of *A. minutum* ranged from 3.6×10^{-5} to 5.6×10^{-5} MU/cell. In the purple clams, the maximal toxicity levels were reached on the last day of exposure period (850 μg STX eq/100 g shellfish meat, 3280 MU/100 g), which was half of the toxicity of previous study (Chen and Chou, 2001). The clams lost 75% of their body burden of toxin on the first day of feeding with nontoxic algae (Fig. 1). The changes in toxicity levels showed a rapid loss of toxicity during the first 6 days of depuration and then the toxicity increased, we suggested that the purple clams had ingested toxic algal cells from pseudofaeces in the sediment. Afterwards, the toxicity decreased from day 48 to day 60 (Fig. 1). By day 60, toxicity levels were still higher than PSP allowable level (80 μg STX eq/100 g (Chen and Chou, 1998)).

Purple clams concentrate toxicity in their digestive glands. During the initial days of feeding, GTX1 was the major toxin in the digestive gland, GTX4 was detected by day 33 and 36 (Fig. 2). In *A. minutum*, GTX1 was the dominant toxins and the toxin profile of shellfish was similar with that of the dinoflagellate. At the end of the exposure period, we found undigested algal cells by

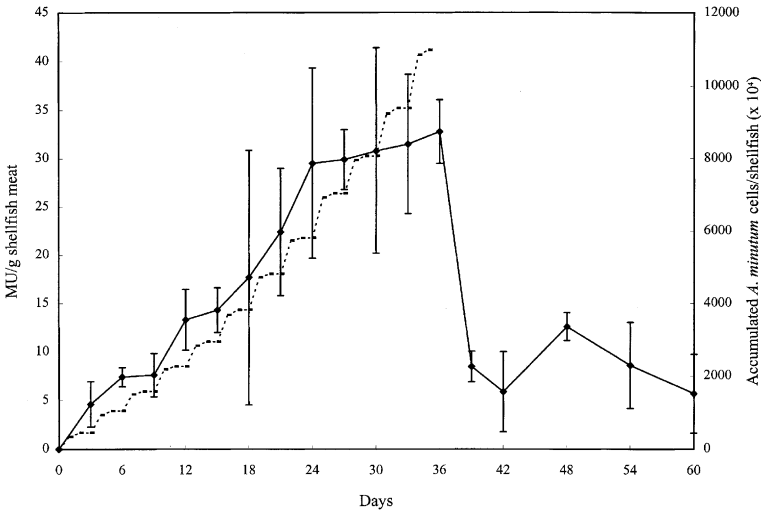

Fig. 1. The amount of ingested toxic dinoflagellate (*A. minutum*) cells (dashed line) and the mouse unit toxicity detected in laboratory cultured purple clams (*H. rostrata*) during the accumulation and depuration period (solid line). Algal cells taken by each of the purple clams were calculated from the daily reduction of the cells. Values of toxicity are means ± SE converted from HPLC analysis of four replications.

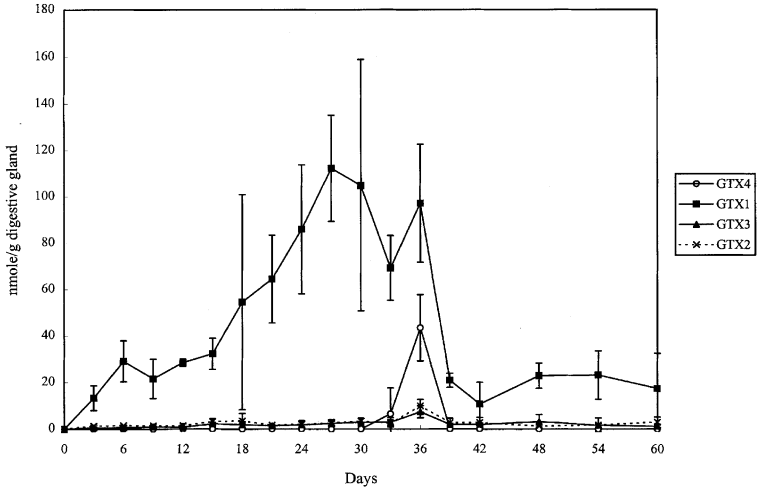

Fig. 2. Changes of PSP toxin compositions in digestive glands of the laboratory cultured purple clams (*H. rostrata*), during the feeding of dinoflagellate (*A. minutum*) and following feeding of nontoxic algae. Values of toxicity are means ± SE converted from HPLC analysis of four replications.

microscopy of the digestive glands of purple clams, so the toxin profile was similar to that of the toxic algae. GTX4 and GTX1 accounted for 88.3% of the molar toxin content in digestive gland. During the depuration period, GTX4 and GTX1 were also the major PSP toxins, the biotransformation from GTX4 and GTX1 to GTX3 and GTX2 might not have occurred (Fig. 2). If we prolong the depuration period and continuously collect samples, GTX3 and GTX2 might become the dominant toxins as the result of our previous study (Chen and Chou, 2001).

In non-visceral tissues, the muscle became toxic by day 6, 12, 15, 33, 36, 39 and 48, the major toxin was GTX1 (Fig. 3). The siphon became toxic by day 12, 15 and 48, the major toxin was also GTX1 (Fig. 4). The results show that muscle and siphon were intermittent toxic during exposure and depuration period. The toxicity which only occurred during depuration period in our previous study (Chen and Chou, 2001) was presumably due to very short half-life of PSP toxins in muscle and siphon.

About the outdoor conditions, the toxicity of outdoor cultured purple clams also concentrated in the digestive gland, and the muscle and siphon were nontoxic during the survey time. The short half-life of GTX3 and GTX2 in outdoor cultured purple clams was similar as that of the laboratory cultured purple clam results. The toxicity level was lower than detection limit (0.518 μg STX eq (2 MU)/100 g) on June 8, 1996. The toxicity level of purple clams were higher than PSP allowable

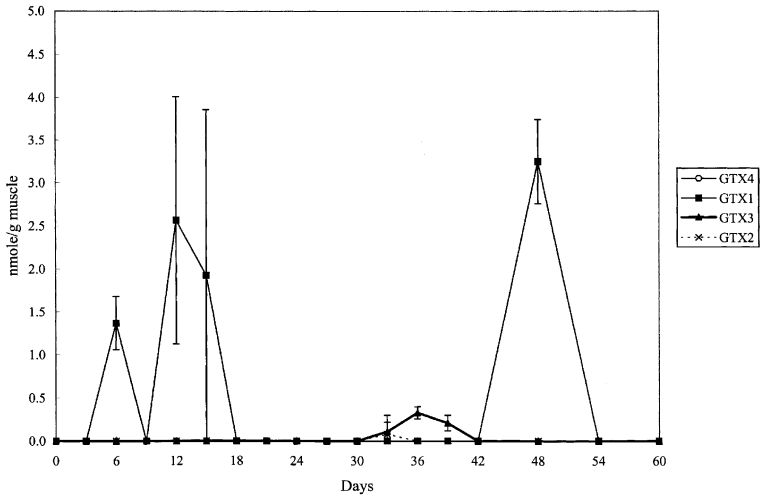

Fig. 3. Changes of PSP toxin compositions in muscles of the laboratory cultured purple clams (*H. rostrata*), during the feeding of dinoflagellate (*A. minutum*) and following feeding of nontoxic algae. Values of toxicity are means ± SE converted from HPLC analysis of four replications.

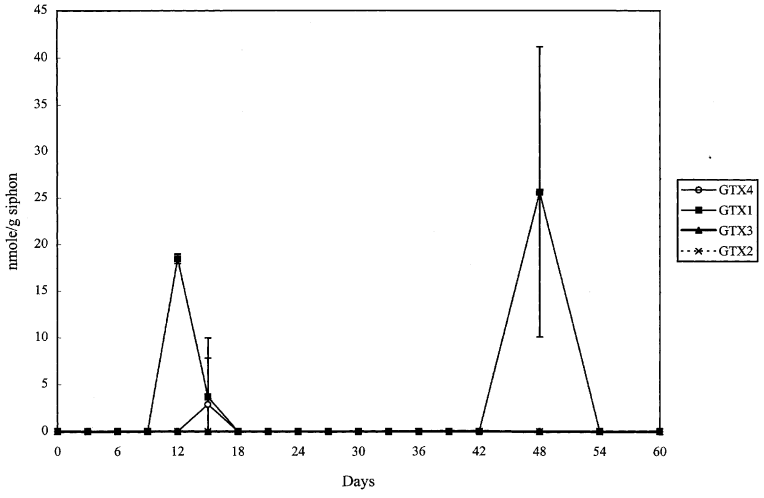

Fig. 4. PSP toxin compositions in siphons of laboratory cultured purple clams (*H. rostrata*), during the feeding of dinoflagellate (*A. minutum*) and the following feeding of nontoxic algae. Values of toxicity are means ± SE converted from HPLC analysis of four replications.

level from June 18 to November 4. Therefore, outdoor cultured purple clams need longer time to decrease toxicity to allowable level. There were two toxicity peaks occurring on June 22 (583 μg STX eq (2250 MU)/100 g) and July 11 (303 μg STX eq (1170 MU)/100 g), and the toxicity of single purple clam attained up to 1372 μg STX eq (5300 MU)/100 g (Fig. 5). Compare the toxin profile between outdoor and laboratory cultured purple clams, the percentage of GTX3 and GTX2 of the former were higher than that of laboratory cultured purple clams (Fig. 6). Furthermore, the toxin profile of outdoor cultured purple clams resembled our field survey of *A. minutum* (the percent of GTX3, GTX2 are higher than GTX4, GTX1). About the number of *A. minutum* in purple clam culture pond, there were only two records (June 6, 1996 (6 cells/ml) and September 23, 1996 (1 cell/ml)). Most of the time, there were no *A. minutum* occurring in the purple clam culture pond during monitoring period.

4. Discussion

In this experiment, GTX4 was transformed to GTX1 quickly after feeding with *A. minutum* cells. In a previous paper, GTX3 and GTX2 were reported to have a longer half-life than GTX4 and GTX1 (Chen and Chou, 2001). However, there is only a hydroxy group difference between GTX4, GTX1 and GTX3, GTX2, that is, the hydroxy group will affect the transformation mechanism in shellfish tissues (Oshima, 1995). Muscle and siphon toxicity could be detected during exposure period, which means that digestive gland transfers toxins to muscle and siphon immediately. The half-life of PSP toxins in

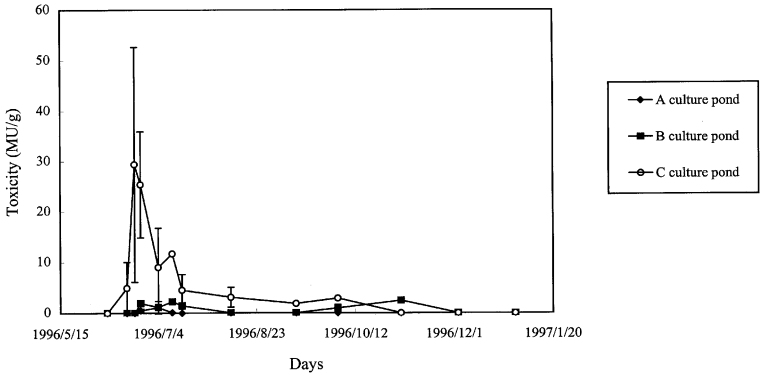

Fig. 5. PSP toxin levels in outdoor cultured purple clams. The purple clams were collected monthly from three close neighbor culture ponds (A, B, and C) from June 1996 to January 1997. Values of toxicity are means ± SE converted from HPLC analysis of two replications.

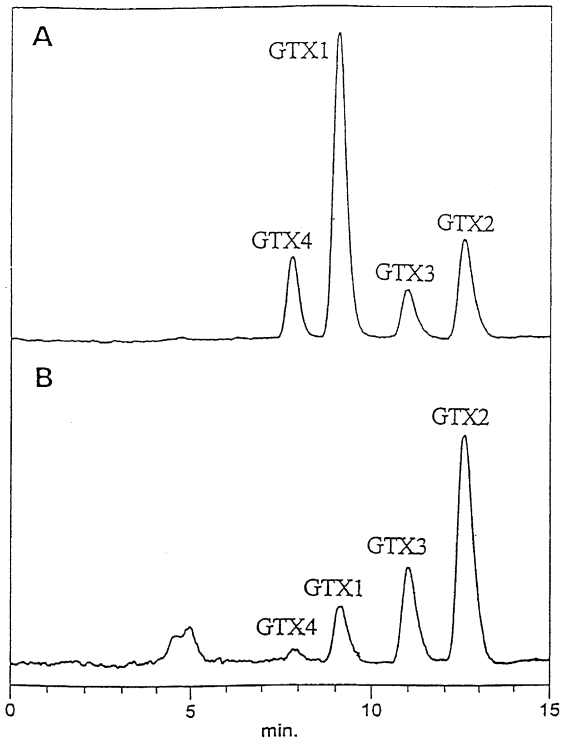

Fig. 6. HPLC chromatograms of PSP toxin profiles of (A) laboratory cultured purple clams (B) outdoor cultured purple clams.

muscle and siphon is short, so toxicity cannot always be detected during exposure and depuration period. The result is different from some studies (Lassus et al., 1996; Bricelj et al., 1990, 1991). Actually, bivalves are open circulation system, there is no distinct septum between muscle and viscera. Therefore, the possibility of man-made contamination should not be excluded. In this experiment, muscle and siphon are intermittent toxic, but the duplicates ($n = 4$) in every collection are similar so exclude the contamination possibility. We found that outdoor cultured purple clams need about six months to detoxify to acceptable levels for human consumption, similar to *Spisula solidissima* (Shumway et al., 1995). Moreover, total PSP toxicity may differ greatly among individual specimens from the same location (White et al., 1993), our study also show the similar results.

In some natural shellfish populations, we could comprehend the toxin origin and toxin transformation mechanism through analyzing the toxicity and toxin profile of toxic algae and shellfish (Shumway et al., 1995). Hallegraeff et al. (1989) found the toxicity of shellfish was high when the concentrations of toxic algae were increasing, and vice versa. In our study, we found PSP toxins in outdoor cultured purple clams, but the number of toxic algae was very low or even not detected in culture pond for most months of the monitoring survey. There might have been a short-term mini-bloom which occurred on June 1996 and September 1996. Furthermore, GTX3 and GTX2 have a longer half-life than GTX4 and GTX1 (Chen and Chou, 2001), so that the outdoor cultured purple clams need longer time to decrease toxicity to allowable level. Other possible reasons to explain the outdoor cultured purple clam toxin accumulation phenomenon are (1) Another unidentified dinoflagellate species, such as *Gymnodinium catenatum* and *Pyrodinium bahamense*, might have produced PSP toxins (Hallegraeff, 1995). However, we did not find any PSP-producing dinoflagellate species other than *A. minutum* in field survey. (2) Purple clams could ingest toxic cysts from sediment and then become toxic. In general, the toxicity of cyst is higher than algal cell (Dale et al., 1978; Lirdwitayaprasit et al., 1990; Oshima et al., 1992). The sediment of culture pond might be toxic due to the release from purple clam (Hwang et al., 1995), and the purple clams could re-accumulate toxicity from the precipitate of *A. minutum* in the sediment. We found few *A. minutum* cysts in the sediment of outdoor culture pond, but the number is too low to analyze the toxicity. Further studies are now in progress in this respect. (3) PSP toxins could be contained by some bacterial strains (Levasseur et al., 1996). The first evidence of PSP toxin production by bacteria was published in 1997

(Gallacher et al., 1997), which demonstrated the presence of GTXs using capillary electrophoresis–mass spectrometry (CE–MS).

The toxin profile of outdoor cultured purple clams resemble the field survey of *A. minutum*, Hwang et al. (1999) also found that GTX3 and GTX2 were dominant in wild cells. However, the toxin profile of laboratory cultured purple clams resemble the stock culture of *A. minutum* (Chen and Chou, 1998, 2001). Some shellfish species could transform PSP toxins to analogues after eating PSP-producing dinoflagellates (Bricelj and Cembella, 1995; Oshima, 1995). It is suggested that the biotransformation of PSP toxins in purple clams is not significant like other shellfish studies. From the results, however, we suggest that laboratory data may not adequately predict times over which pond-cultured purple clams may prove toxic to consumers.

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