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Determination of PSP Concentration in Shellfish From Kuala Penyu, Sabah Using HPLC Method

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ABSTRACT

Pyrodium bahamense var. compressum is the main causative algae that causes paralytic shellfish poisoning (PSP) in Sabah. The most recent event occurred in 2013 when toxin levels of between 600 and 800 Mouse Unit (MU) were detected using the mouse bioassay method. However, the mouse bioassay method is not able to distinguish between the different PSP analogues and its concentration. This study was carried out to determine the PSP concentrations in shellfish 2 years after the bloom occurred using analytical methods. Two different species of shellfish (*Perna viridis* and *Geloina* sp) were collected from Tasek Sitopmok, Kuala Penyu, Sabah. Toxin from that shellfish tissues were extracted using 0.2 M HCL and analyzed using High Performance Liquid Chromatography with post column fluorescence detector (HPLC-FLD). Results showed that tissue samples extracted from *Geloina* sp were free from any contamination of PSP toxin. Meanwhile *Perna viridis* extracted sample, showed the presence of PSP toxin (decarbamoyl derivatives) which is Gonyautoxin (GTX 4), with toxin content of 30µgeq/100g tissue. Regularly monitoring for PSP toxin is required using analytical methods such as the HPLC due to its capability to express the actual toxin concentration as well as being able to distinguish the different types of toxin derivatives.

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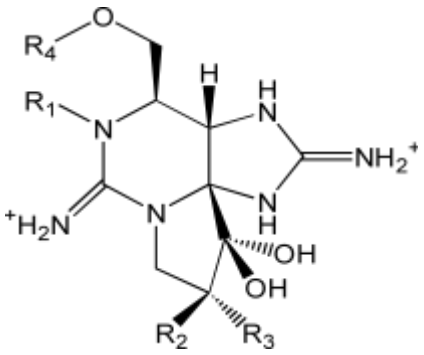
Introduction

Pyrodium bahamense var. compressum is the main causative algae that causes paralytic shellfish poisoning (PSP) in Sabah. This marine bio-toxins are highly potent due to its capability block the sodium channel of the neuron cell membrane and lead to sudden death and paralyzed (Long *et al.*, 1990). PSP are typically comprised of saxitoxin and its derivatives that grouped into three categories which are carbamate toxin (highly potent), decarbamoyl toxins (intermediate potent) and *N*-sulfocarbamoyl toxin (least potent) (Shimizu & Yoshioko, 1981; Lasus *et al.* 2000). The diverse composition of saxitoxin and its derivatives as shown in Figure 1 below. There are four functional groups (R1-R4) that function to determine the potential of STX toxicity.



R1	R2	R3	Carbamate	*N*-Sulfocarbamoyl	Decarbamoyl
			R4 (functional group)		
			–CONH2	–CONHSO3—	H
H	H	H	STX	GTX5	dcSTX
OH	H	H	neoSTX	GTX6	dcneoSTX
OH	OSO	H	GTX1	C3	dcGTX1
H	OSO	H	GTX2	C1	dcGTX2
H	H	OSO	GTX3	C2	dcGTX3
OH	H	OSO	GTX4	C4	dcGTX4

Figure 1. Structure of saxitoxin and related derivatives
(Sources: Oshima, 1995 ; Smith *et al.*, 2001).

The most recent event occurred in Sabah was reported 2013 when toxin levels of between 600 and 800 Mouse Unit (MU) were detected using the mouse bioassay method (Sabah Fisheries Department, 2013). However, this mouse bioassay method is not able to distinguish between the different PSP analogues and its concentration. Moreover, excessive killing of mice is required and against animal ethics (Tan & Ransangan, 2014). Therefore, analytical method based on chromatography analysis is developed to replace mouse bioassay (Sullivan *et al.*, 1985 ; Nagashima *et al.*, 1987). Recently, High Performance Liquid Chromatography (HPLC) system proposed by Oshima (1995) quite popular because this system capable to separate all PSP toxins including saxitoxin and it derivatives (Chen & Chou, 2002). This study was carried out to determine the PSP toxicity level in shellfish 2 years after the bloom occurred using high performance liquid chromatography (HPLC).

Methodology

Sample collection

Twenty individuals of 2 different species of shellfish (*Perna viridis* and *Geloina* sp) were collected from Tasek Sitompok, Kuala Penyu, Sabah. *Perna varidis* which averaged 6.5 cm (S.D ± 0.53 cm) in total length and 30g averaged tissues wet weight (S.D ± 3.24g) while *Geloina* sp which averaged 4.5cm (S.D ± 0.35g) and 15.6 g mean tissue wet weight (S.D ± 0.48 g) were thoroughly cleaned and stored at -20°C before toxin extraction.

Toxin extraction

Toxin from shellfish tissues was extracted according to AOAC, (2003) methods. Shellfish tissues were homogenized in 0.1 M acetic acid at room temperature at moderate acidity (pH 4 to 5) and then heated in 100°C water bath for 20 minutes. The homogenate was centrifuged at 1, 500 g for 20 minutes and the supernatant obtained was filtered through Sep-Pak C18 filter cartridge that has first been conditioned with deionized water and 100% methanol. The toxin eluent from cartridge was collected inside 5 ml vial and stored in -20°c before analysis.

Toxin Analysis

Extracted toxin was analyzed using Shimadzu High Performance Liquid Chromatography with post column fluorescence detector (HPLC-FLD) system following the method suggested by Oshima (1995). Toxin separations were analyzed using reversed phase column, 5 µm, 250 x 4.6 mm Supercoil C18 column. HPLC condition used for this analysis is summarized in Table 1. Toxicity of each shellfish was expressed as µgeq/100g tissue, which was calculated from nmol g-1 obtained by HPLC analysis using specific toxicity values of each toxin component (Ontojo *et al.*, 2012).

Table 1: HPLC conditions used for the analysis of PSP toxins according to Oshima (1995)

Parameter	**Condition or description**
Column	Reversed phase C18-bonded silica gel (250 x 4.6 mm)
Mobile phase	
Flow rate	0.8 ml/min
For GTX 1-4	Sodium 1-heptanesulfonate (2 mM) in 10 mM ammonium phosphate, pH 7.1
For STX	Sodium 1-heptanesulfonate (2 mM) in 30 mM ammonium phosphate, pH 7.1
Oxidizing reagent	
Flow rate	0.4 ml/min
Composition	Periodic acid (7 mM) in 50 mM potassium phosphate buffer, pH 9.0
Reaction	10 m Teflon tubing (0.5 mm i.d.) at 85°C in a drying oven
Acidifying reagent	
Flow rate	0.4 ml/min
Composition	0.5 M acetic acid
Detection	
Excitation	330nm
Emission	390nm

Result and discussion

Results showed that tissue samples extracted from *Geloina* sp were free from any contamination of PSP, while sample extracts from *Perna viridis*, showed the presence of PSP toxin (decarbamoyl derivatives) which is Gonyautoxin (GTX4), with toxin content of 30 µgeq/100g tissue (Figure 2). There is possibility that *Perna varidis* accumulate toxin from the previous *Pyrodinium bahamense var. compressum* blooms and stored/bond inside tissues as decarbomyl derivatives and/or other saxitoxin derivatives up to 2 years' periods before released it as waste. Study done by Sekiguchi *et al.* (2001) shellfish species like mussel showed PSP toxin accumulation when exposed to toxin dinoflagellate, *Alexandrium tamarense* both in the wild and fed in the control tank. However, the ability of shellfish to sustained PSP toxin inside their tissue vary among shellfish species (Deeds *et al.*, 2008). Twarog (1974) proposed that previous history of exposure to PSP may also affect shellfish PSP toxin accumulation, such that shellfish populations repeatedly exposed to PSP toxins might become more resistant and concentrate higher toxin levels than those with no experienced contamination.

Although considered safe for human consumption, GTX-4 can undergo bio-transformation when chemical and enzymatic reaction changes the toxin concentration from less potent to highly potent derivative. This bio-transformation depends on environmental factors such as pH and temperature (Bricelj & Shumway, 1998). However, at which temperature affects the uptake and release of PSP toxin is not clearly defined (Madenwald, 1985).

Major PSP toxin in marine shellfish undergoes bio-transformation in the digestive gland, indicating the presence of enzyme that transform PSP derivatives to another form of PSP derivative (Fast *et al.*, 2006). This transforming enzyme required specific pH and temperature (Bricelj & Shumway, 1998). Previous study done by Sullivan (1982) found that conversion of GTX 5 to STX is possible in blue mussel. Moreover, toxin extracted from marine crabs, snails and red algae have been also transformed GTX 1-4 to STX or/and neoSTX (Sugawara *et al.*, 1997; Kotaki 1989).

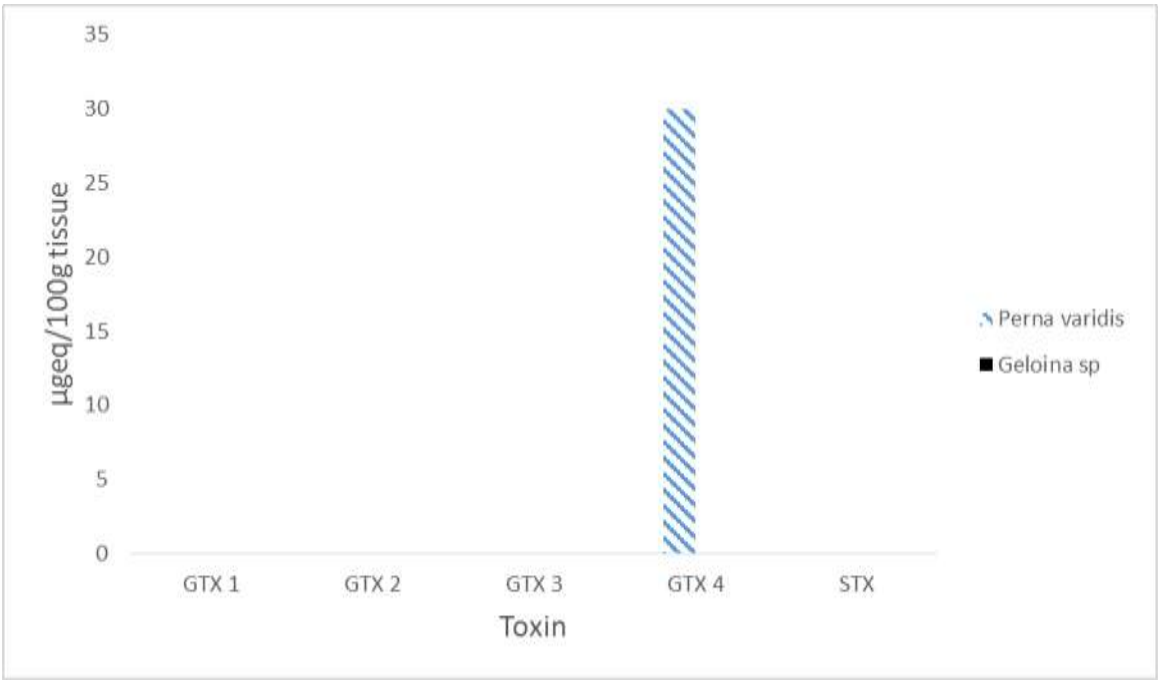


Figure 2. PSP toxin content in *Perna varidis* and *Geloina sp*

Conclusion

This preliminary study proved that presence of PSP toxin in *Perna varidis* sample collected from Tasik Sitompok Kuala Penyu. This PSP toxin has tendency to become highly potent when undergoes biotransformation. The results presented here may facilitate improvements in the PSP toxin regulatory guidelines in Sabah. The remarkable result in accumulation of PSP toxin in 2 shellfish species indicates shellfishes can become reservoirs of PSP toxin. Further study using HPLC is needed in order to get more precise data since HPLC is able to measure the toxin concentration as well as to distinguish between the different types of toxin derivatives.

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