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Original Paper

SAXITOXIN IN GREEN MUSSELS (*Perna viridis, Mytiliae*), BLOOD COCKLE (*Anadara granosa*) AND FEATHERS COCKLE (*Anadara antiquata, Arcidae*) USING HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

Saxitoxin (STX) was measured in green mussels (Perna viridis), and feathers cockle (Anadara antiquata, Arcidae) from Jakarta and blood cockle (Anadara granosa) from Jakarta and Indramayu. Samples were taken 7 times from fish market Muara Baru Jakarta and Karangsong Indramayu. All samples were collected from June up to October 2009. The aim of this research is to find out the content of STX in the mussels. The mussels tissues were homogenized, weighed and extracted with 0.1 M HCl. The supernatan were filtered by 0.45 µ nylon membran. Fluorescence oxidation of STX was carried out using 2% H_2O_2 in alkaline solution. Analysis was performed using HPLC equipped with a C18 column (4.6 mm×250 mm, 5 µm), fluorescence detection (ex 340 nm, em 400 nm) elution of acetonitrile/0.1 M ammonium formate solution (5:95, v/v, pH 6) at a flow rate of 1.0 ml/ min. The calibration graphs was prepared by injecting standards ranging from 0,5–20 ng/ml, giving an acceptable linearity (r = 0.999). Retention time of saxitoxin standard was detected at 5.467 min. Negative results were obtained for most of blood mussels from Indramayu with the exception of the 4th and 7th sampling. Saxitoxin were detected in Perna viridis and Anadara antiquata, Arcidae ranging from 0.87–5.39 µg/ 100 g and 0.14–0.9 µg/ 100 g wet tissues respectively.

Keywords: Saxitoxin,; PSP toxin ; green mussels (*Perna viridis, Mytilidae*) ; blood cockle (*Anadara granosa*) and (*Anadara antiquata, Arcidae*)

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INTRODUCTION

Saxitoxin (STX) is a highly toxic compound, i.e., 100 times more toxic than strychnine, 1000 times more toxic than the synthetic nerve gas sarin, and 2000 times more toxic than sodium cyanide. Pigs as experimental animals, died when injected intra-muscularly at a dose of 5 g/kg and at the same dose, mice will die by intra-peritoneal injection. Poisoning of STX can be through digestive and respiratory routes. Poisoning through inhalation, causes respiratory failure so that death can occur within 2-12 hours and there is no cure. Aid treatment can be conducted by giving artificial respiration. Poisoning through the digestive system occur when consuming shellfish which have been contaminated naturally by STX. People are poisoned quickly by this toxin, with a syndrome known as PSP (Paralytic Shellfish Poisoning). The symptoms include burning sensation on the tongue, lips and mouth, which then spread to the neck, arms and legs. Patient may go through numbness, so that movement becomes difficult. In severe cases followed by a feeling of floating, drooling, dizziness and vomiting (Hall *et.al*., 1990).

Saxitoxin (STX) has a molecular structure as seen in **Fig. 1.** This compound has the chemical name (3aS-(3a-α,4-α, 10aR *)) 2,6-diamino-4-(amino-carbonyl) oxy) methyl) -3a,4,8,9-tetrahydro-1H,10H-pyrrolo (1,2-c) purine 0.10-10-diol with molecular formula $C_{10}H_{17}N_7O_4$ and has a molecular weight of 299.29 g mol-1 (Hall *et.al*, 1990).

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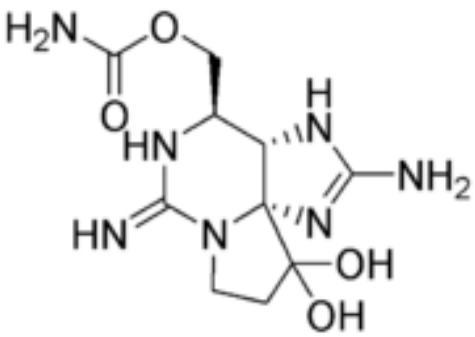

Fig. 1. The structure of saxitoxin (Hall *et.al*, 1990).

As a natural neurotoxin, saxitoxin is produced by certain marine species of Dinoflagellata (*Alexandrium* sp., *Gymnodinium* sp., *Pyrodinium* sp.) and Cyanobacteria (*Anabaena* sp., some of *Aphanizomenon* spp., *Cylindrospermopsis* sp., *Lyngbya* sp., *Planktothrix* sp.). Dinoflagellates thrive in warm water environment with low salt concentrations, abundant light. In this environment dinoflagellates might in a particular condition, grow in large numbers, so that the color of sea water becomes red, this phenomenon known as red tide (Funari and Testai, 2008; Anonim, 2004). In various regions of Indonesia waters blooming of this type of dinoflagellates often occurred and in general calaled as HAB (Harmfull Algal Blooms). Some studies have carried out on periodic monitoring of phytoplankton distribution of HAB species in Indonesian waters *e.g* Pulau Panggang (Kepulauan Seribu), Jakarta Bay in 2008, and the Makassar Strait in 2004 (Thoha, 2008). The results showed that in these areas dinoflagellate bloom have been frequent.

Mussels which are filter feeder, can serve as bio indicators to determine whether marine biota have been contaminated by PSP toxins. Therefore, a periodic monitoring is a must. STX has a high toxic nature, therefore, specific analytical methods, which should be sensitive and rapid is needed to assess the existence of STX in the mussels. Analysis of STX in Indonesia usually carried out by using mice bioassay (Mulyasari *et al.*, 2003). This method has a minimum detection limit 40 ug STX/100 g wet mussels. Many countries such as China, America and Canada have a strict standard for PSP in shellfish i.e, amount of PSPs should not exceed 80 µg/100 g, while in the Philippines the amount of PSP cannot be higher than 40 µg/100 g wet weight (Jaimea, 2001).

Today many researchers have developed analytical methods of STX in seafood using High Pressure Liquid Chromatography (HPLC) with fluorescence detector, ion exchange and mass spectrometry (Lawrence *et al.*, 2005; Cianca *et al.*, 2007; Oshiro *et al.*, 2006). Di *et al.*, 2006, used the Jellett rapid method to detect positive or negative test for PSP toxin in shellfish samples. In this study, STX in shellfish samples is analyzed by means of HPLC using fluorescence detector, where STX is oxidized using H_2O_2 prior to be analysed. The method is the modified from Di *et al.*, (2006).

Bio assay was neither sensitive nor selective, therefore, it was mainly used as a screening method. LC / MS (Liquid Chromatography Mass Spectrometry) provided structural information of compounds, which was a confirmatory method. Due to the lack of chromophore in the molecule of PSP (**Fig. 1**), it cannot be directly observed by ultraviolet or fluorescence detector. So the PSP molecules need to be oxidized to be detected with fluorescence detector (Di *et al.*, 2006).

Analysis was carried out at Earth and Environmental Laboratory, Centre for the Application of Isotopes and Radiation Technology, National Nuclear Energy Agency Jakarta. The aim of the research is to find out the content of STX in the *Perna viridis*, *Anadara granosa* and *Anadara antiquate, Arcidae.* It is expected that this method can provide assurance that the Indonesian mussels

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are safe for public consumption, as well as for domestic and export purposes.

MATERIAL AND METHODS

Sampling

Anadara granosa obtained from fish auction markets in Muara Baru Jakarta and Karangsong, Indramayu, whereas *Anadara antiquate, Arcidae* and *Perna viridis* were obtained from fish auction market in Muara Baru Jakarta. Sampling was conducted 7 times between June and October 2009 forth night interval. Samples in a whole were stored in a cool box (±4^{o}C) until STX extraction.

Saxitoxin dihydrochloride as a standard material with a concentration of 65 ± 3 µmol/l at 20°C was obtained from the Institute for Marine Bioscience (NRC) California, Acetonitril HPLC grade (JT Baker), Ammonium format of HPLC grade (Sigma-Aldrich), HCl, NaOH, glacial acetic acid and hydrogen peroxide 30% by Merck and pro-quality analysis were all among the chemicals used.

Extraction.

Samples were washed, opened to separate shells from the meat, which then were thawed and weighed. The average wet weight of tested meat for the *Perna viridis*, *Anadara granosa* and *Anadara antiquate, Arcidae* were 2.5; 5.13 and 1.7 g, respectively. A total of 100 g of mussels meat, are blended and 10 g of mussels meat then put in a 50 ml polypropylene tube screw lids. Then, add 10 ml of 0.1 N HCl, shaken with a vortex, and adjusted the pH 3-4 by dropwise addition of 1 N HCl or 0.1 N NaOH. The tube was closed tightly and put in a beaker of boiling water for 5 minutes. The tube are removed, cooled and the pH of the sample are adjusted again to 3-4. The samples were centrifuged at 3000 g for 15-20 minutes. Supernatant was filtered with nylon size 0, 22 µm and stored at -20^{o}C. In this condition the samples can be stored for 6 months prior to the STX concentration test.

Determination of STX in the sample by HPLC (Lawrence et al., 2005, Anonim, 2000)

This method was based on the precolumn oxidation where STX were oxidized using H_2O_2. Amount of 100 µL sample solution or STX standard were transferred into 1.8 ml a plastic centrifuge tube covered with aluminum foil. 275 µL of 2 % alkaline H_2O_2 were added and mixed The mixture were reacted for 3 min at 20^{o}C, and finally 20 µL of glacial acetic acid were added to terminate the reaction. A total of 20 µL of this solution were injected into HPLC with Fluorescence Detector (Waters, 470) associated with C-18 column, phenomenex (250 x 4.6 mm2 and particle size 5 µm), analytical balance, centrifuges, blenders, micropipettor (1000-1000 mL), vortex, and 0.45 µm membrane filter size.
Calibration curve was obtained by oxidizing standards at concentration 0.5-20 ng/mL. Recovery test was conducted by taking 500 mL of standard STX 400 ng/ml, into a 5 g test sample, stirred and allowed to stand for 5 minutes. STX in the test sample was extracted and the content was analyzed in the same procedure as sample.

Chromatographic conditions.

a reserved-phase column C-18, phenomenex with dimensions of 250 mm x 4.6 mm, 5 µm particle size was used for separation of the toxin oxidation products. The mobile phase was acetonitrile in 0.1 M ammonium formate (5:95, v/v), pH of mobile phase was adjusted to 6 with 0.1 M HCl. Then the mobile phase was degassed by ultrasonic for about 30 min, and was kept at 4^{o}C in dark place. Flow rate 1.0 ml/min. Fluorescence detection (ex λ 330 nm, em λ 390 nm).

RESULTS AND DISCUSSION

Limit detection and calibration curve of STX

Based on the experiment, concentration of saxitoxin standard is 0.5 ng/mL. Injection was carried out at concentration below 0.5 ng/mL, *i e.* 0.2 ng/mL, injection of saxitoxin standard at concentration 0.20 ng/mL resulting no peak, it is concluded that limit detection of STX standard by gas chromatography is 0.5 ng/mL,

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this value is equivalent to 0.1 g STX/100 g wet sample.

Calibration curve was made by injecting 20 µL STX standard at varied concentration, *i e* ; 0.5, 1; 2; 4; 10 and 20 ng/mL. A good linearity was obtained at a standard concentration of STX between 0.5 to 20 ng / ml with R^2 = 0.9990 and the linear equation Y = 9778 X-41.45. The calibration curve shown in **Fig 2.**

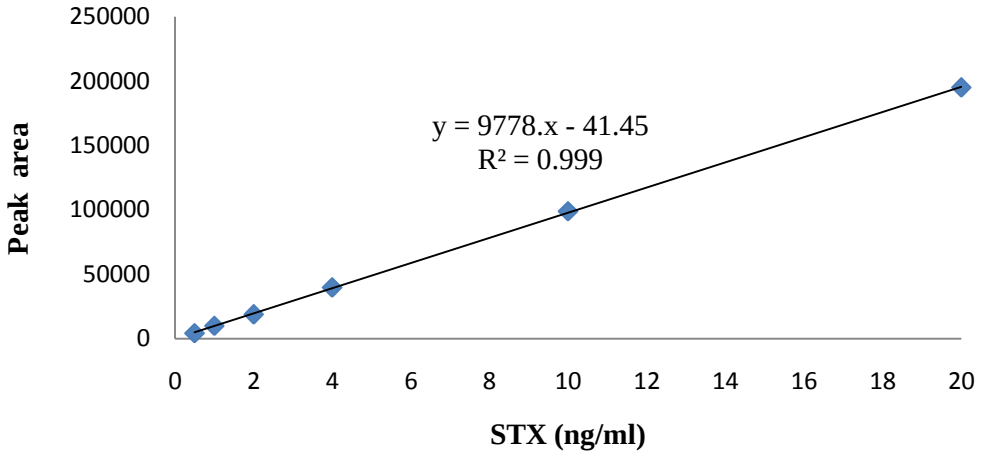

Fig. 2. Calibration curve of STX standard at concentration of 0.5-20ng/ml

Chromatogram of blank and STX standard (10 ng) could be seen in **Fig. 3a** and **3b**, respectively. X-axis represents time (minutes) and the Y-axis is the electrical signal that is expressed in mV. Magnitude of the detected electrical signal is converted into the amount of peak area and is proportional to the concentration of substances analyzed. Chromatogram of blank (**Fig.3a**), resulting in no peak at retention time of 5.467± 0.1 min, but chromatogram of saxitoxin standard has a peak at retention time 5.467± 0.1 min (**Fig.3b).** It is therefore indicated that saxitoxin standard has a retention time at 5.467± 0.1 min. Intensity of the peak indicated the concentration of STX standard, the higher the intensity the higher the concentration.

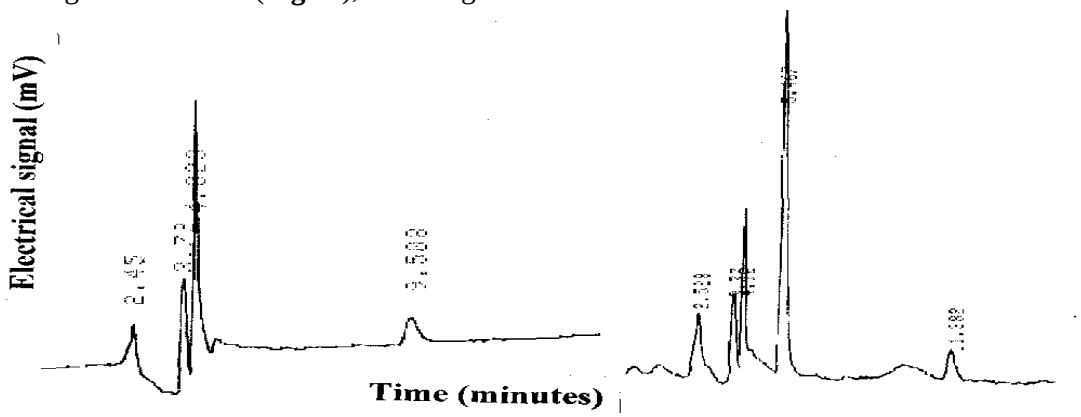

Fig. 3.a. Chromatogram of blank

Fig. 3b. Chromatogram of STX standard (10 ng/ml)

Recovery test.

Recovery test was conducted by taking 500 µL of STX standard 400 ng/ml, into a 5 g test sample, stirred and allowed to stand for 5 minutes. STX in the test sample was extracted and analyzed by the same procedure as sample. Recovery of STX in *Perna viridis* resulting 68%.

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STX in mussels sample

Saxitoxin content in *Anadara granosa* from Muara Baru Jakarta and Karangsong Indramayu showed in **Table 1.** Negative results were obtained for most of *Anadara granosa* from Indramayu, but detected at sampling 4th and 7th, the concentration were 0.91 and 0.49 (µg/100 g wet weight). The saxitoxin concentration from Jakarta were 0.33; 0.20; 0.61; 0.31 and 0.37 (µg/100 g wet weight) at 1st, 4th, 5th, 6th and 7th samplings, respectively. The highest concentration of saxitoxin, of 0.91 µg/100 g wet weight, was taken from Indramayu, yet still below the threshold value that recommended from European Community (EC).

Table 1. Concentration of STX l in *Anadara granosa* from Jakarta and Indramayu

Sample	Concentration of STX (µg/100 g wet weight) in *Anadara granosa*	
	Jakarta	**Indramayu**
1	0.33	not detected
2	not detected	not detected
3	not detected	not detected
4	0.20	**0.91**
5	0.61	not detected
6	0.31	not detected
7	0.37	0.49

* Detection limit of HPLC is 0.1 µg/100 g wet sample

The incident in Jakarta more frequent compare to that in Indramayu, although the values below the threshold recommended from European Community. According to Thoha (2008), the waters of Jakarta Bay frequent and often occur of mass mortality in this region. Levels of pollution in marine environments in Indonesia were still high with the increasing amount of nutrients caused by pollutants excessive. Nutrition *e.g* generally derived from domestic wastes such as detergents, and agricultural activities in watersheds that enter the sea (Lestari dan Edward, 2004). Pollution at sea can also be characterized by an increased growth of phytoplankton/algae excessive and tend to decompose faster. Pollution in the marine environment called red tide caused by phytoplankton blooms which will gave a negative impacts on many coastal waters, particularly those being utilized for seafarming (Corales and Maclean, 2000).

A. granosa can live in 20m water depth but concentrates in the littoral area (time for ground drying: 6 - 10 hs/day and night) (Tran, 1977) *A. granosa* is a typically intertidal species which naturally lives in an area of silty bottom with relatively low salinity and some time of desiccation (when ebbing) every day (Kuang and Sun, 1995). Shallow Burrower. Filter Feeder. Their feeding habit is related to the bottom feed where they live. Their important nutrient components are organic detritus (98% were found in cockle's intestine), phytoplankton and unicellular algae (Tran, 1997).

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Table 2. Saxitoxin content of STX from *Perna viridis* and *Anadara antiquata* from Jakarta

Sample	STX std (µg/100 g wet weight)	
	Perna viridis	***Anadara antiquata, Arcidae***
1	3.59	0.51
2	3.46	0.50
3	3.12	0.25
4	2.21	0.17
5	5.39	0.90
6	2.82	0.3
7	0.87	0.14

The saxitoxin content from *Perna viridis* and *Anadara antiquata* from Jakarta were seen in **Table 2.** The saxitoxin content in *Anadara antiquata* lower than that in *Perna viridis* it were ranging from 0.14 to 0.9 µg/100 g wet sample. The highest saxitoxin concentration detected in *Perna viridis* at sampling to 5th (5.39 µg/100 g wet sample), the other samples were ranging from 0.87 up to 5.39 µg/100 g wet sample. Although the value is high, but still below the threshold value that recommended from European Community (EC). According to European Community (EC) regulatory limit 80 ug STX equivalents (eq) 100 g^{-1} of shellfish flesh are recorded using the mouse bioassay (Stobo *et al.*, 2008).

Mussels, ecologically they play a key role in food chains, consuming plankton and other filter food and being consumed by fish, birds, marine mammals, other vertebrates and various invertebrates. They also help to filter water, being natural water purifiers and are indicators of water pollution (www.newworldencyclopedia.org). *Perna viridis* are coastal bivalves, typically occurred at depths of less than 10 m, and shown to be tolerant to wide range of turbidity and pollution (Power *et al.*, 2004). *Perna viridis* typically occurs at depths of less than 10 metres and inhabits intertidal, subtidal and estuarine environments and is often found in densities as high as 35,000 individuals per square meter in any submerged marine object. While the mussels usually attach to hard substrata they are capable of relocating and can colonize man made habitats such as bridges, pier pilings, sea walls, boys, boats, etc.

Anadara antiquata can grow well in zone littoral waters and sublitoral with the type of quiet waters, particularly in sandy and muddy bays to a depth of 30 m, but are commonly used as a place to live is the littoral areas where the area is still affected by tides (Poutiers, 1998). The results of the present study provide further evidence that the accumulation of saxitoxin in *Perna viridis, Anadara granosa and Anadara antiquata* caused by marine environment red tide.

Example chromatograms of *Perna viridis* samples containing saxitoxin can be seen in **Fig. 4.** The chromatograms of *Perna viridis* samples has a retention time of 5.505 min. By comparing this chromatogram with chromatogram of STX standard with retention time 5.467 min (**Fig. 3b**), it is assumed that chromatogram of **Fig. 4** containing saxitoxin.

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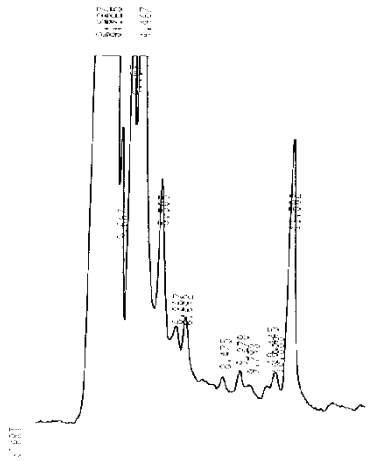

Fig. 4. Chromatogram of *Perna viridis* sample

CONCLUSION

From this study it can be concluded that saxitoxin in bivalvesamples can be detected by HPLC using fluorescence detector after oxidized using hydrogen peroxide. Limit detection obtained by 0.1 g STX/100 g wet tissue weight. Negative results of saxitoxin was obtained from most *Anadara granosa*. The highest saxitoxin was obtained from *Perna viridis* ranging from 0.87 to 5.39 µg/100 g wet sample with highest concentration of 5.39 µg/100 g wet sample. The saxitoxin content in *Anadara antiquata* ranging from 0.14-0.9 µg/100 g wet sample.

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