Acetylcholinesterase (AChE): Potential Biomarker for Evaluating Pesticide Exposure on Egg and Tissue of Golden Apple Snail (*Pomacea Canaliculata*) from Huai−Saneng Reservoir, Surin Province, Thailand

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ABSTRACT

The aim of this study was to evaluate pesticide exposure in egg and tissue of golden apple snail *(Pomacea canaliculata*), being collected from Huai−Saneng Reservoir, Surin Province by using AChE as bio−indicator. This is the pioneer work in Thailand with regards to the application of Acetylcholinesterase (AChE) as a situ biomarker in indicating pesticide contamination. The snail and its egg were sampled two times in the period of rice cultivating in June and July, 2017. There were 5 sampling stations (n = 10). The snail was classified based on its sizes: small, medium, and large. After studying the protein form by using 12.5% SDS−PAGE technique, it was that found that there were differences in protein expression from post−fertilization egg (pink color) and pre−hatching egg (white color). The results of Western blot analysis indicated that AChE of the egg in pink colored stage had 2 isoforms with different weights: 71 kDa and 66 kDa. However, there was only 71 kDa of AChE of that in white colored stage. The protein in 3 snail sizes was not different when compared to the snails from 2 sampling periods. Moreover, AChE expression in collected snail was higher than that in controlled group with having only 1 isoform (71 kDa). The AChE expression in the snail from stations 4 and 5 were lower than that from the other stations. After organochlorine and carbamate pesticide contamination in water was studied using test kit, it was found that there was contamination in every station in both periods and the highest was found in stations 4 and 5. Based on our results, we concluded that AChE expression in the egg and tissue of golden apple snail has a high potential to be used as an early warning signal indicating a pesticide contamination in the environment.

Keywords: Acetylcholinesterase, biomarker, pesticide, golden apple snail: *Pomacea canaliculata*

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INTRODUCTION

As generally known, pesticides have been increasingly applied in agricultural areas and in some cases their applications are excessive and misused. Moreover, some negative effects were neglected by farmer. Thus, they can cause serious health and environmental problems into both human and animals. The most important sink of these pesticides is waters because they are the end of every water way. The pesticides in aquatic environments can influence the water quality, and potentially have adverse effects on drinking water quality and biodiversity (Tufi *et al*., 2016). In 1995, Pimentel *et al*. (1995) reported that only 0.1% of applied pesticide affected targeted organism while the remaining contaminated the environment: water, soil and air.

The freshwater snail is classified as the primary consumer in aquatic food chain. They may expose to contaminated pesticides by both feeding and assimilation. They may feed on plankton, plant and rice trunk that contaminated with pesticides. Besides, they may assimilate pesticides being dissolved in water, adsorbed on particulate or bottom sediment. Moreover, they mostly live in stagnant waters making riskier to expose to pesticides and to accumulate them in their body. In addition, these pesticides can reach human beings through food chain. USGS (2018) also indicated that aquatic organisms such as fish, edible fish, and mollusks, play an important role in transporting contaminants, especially hydrophobic ones to the top consumer of food chains both human being and wildlife. The freshwater snail was commonly found in many aquatic environments; however, the study on applying it as bio−indicator has been inadequate. This may be due to lack of any guideline to assess. However, many studies reported that the snail can be used as an appropriate indicator to evaluate water and sediment quality (Tallarico, 2015). In addition, many reports indicated that they can be used to evaluate the contamination of other chemicals such as heavy metals, tributyltin, and insecticide (Piyatiratitivorakul and Boonchamoi, 2008; Putkome *et al*., 2008; Tallarico, 2015; Martínez *et al*., 2017) because they mostly move slowly beneath water surface and embed in the mud.

Golden apple snail (*Pomacea canaliculata*) is classified in the group of freshwater snail. It origins in Africa and then spreads into Asia (Dai *et al*., 2011). As generally known, golden apple snail is an invasive alien species which widely spreads nationwide. It can be found in paddy field or agricultural area and cause severe damage on farming because they feed on rice trunk or other aquatic plants. Thus, farmers have to apply pesticides in their fields resulting in accumulation in surrounding environments (Putkome *et al*., 2008). Many reports indicated that most pesticides have been applied for increasing plantation efficiency. The effect of many pesticides is to inhibit Acetylcholinesterase (AChE) that is neuron transmitter in the case of long−term exposure. However, the exposing organism is stimulated to synthesize more AChE in the short−term exposure. Thus, they suggested that AChE should be used as environmental bio−indicator. Walker *et al*. (2006) indicated that AChE specified to organophosphate and carbamate pesticides thus it could be used as early warning signal. Recently, a report indicated that glyphosate which is a herbicide having negative effects onto the health and moreover being magnified through aquatic food chain composing of protozoa, mussels, crustaceans, frog, fish and finally human. This phenomenon is similarly found in terrestrial animals (Van Bruggen *et al*., 2018). Based on its serious effect, it thus should be further studied.

In Surin province, Huai−Saneng Reservoir is very important to local residents and nearby area in both food security and economy. It was reported that fishery in that reservoir can be performed all year round because it is very fertile. Moreover, water quantity is enough to supply for agricultural area. Therefore, pesticides that reach into the reservoir may accumulate and pass through local people. This study aimed to evaluate pesticide exposure in egg and tissue of golden apple snail using AChE as bio−indicator. The results achieved can be used as a guideline for environmental management for

both water and aquatic organism. And, it was also applied to decrease pesticide application. Moreover, it can be used as fundamental knowledge for the local people who often consume golden apple snail (*P. canaliculata*) or other aquatic organism.

MATERIALS AND METHODS

Collection of Golden Apple Snail and Its Egg

Golden apple snail and its egg were collected from Huai−Saneng Reservoir, Surin Province. They were sampled two times in rice farming period in June and July, 2017. There were 5 sampling stations covering the reservoir. The station 3 was in the center and stations 1 and 2 were 2.5 and 5 km northward, respectively. The stations 4 and 5 were in southward (Figure 1). The snail (n = 10) sampled from each station was classified into 2 stages based on egg color: pink (post−fertilization egg) and white (pre−hatching egg) (Figure 2). The size of egg was about 4.1 ± 0.8 cm with weight of 7.3 ± 1.1 g. The snail was also classified as 3 groups: small (15 ± 2.1 g), medium (24 ± 2.8 g), and large (67 ± 5.5 g).

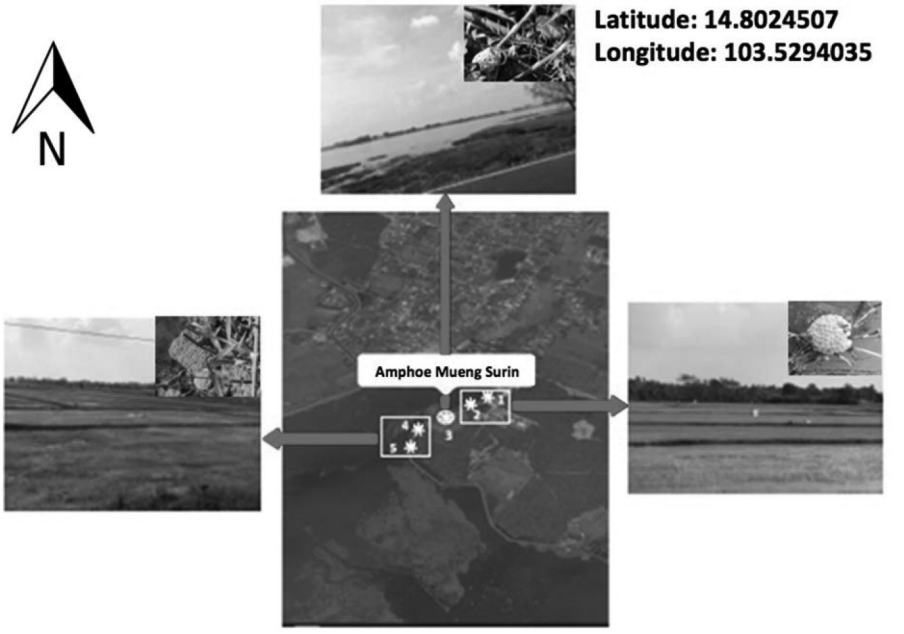


Figure 1 Sampling station at Huai−Saneng Reservoir, Surin Province, Thailand

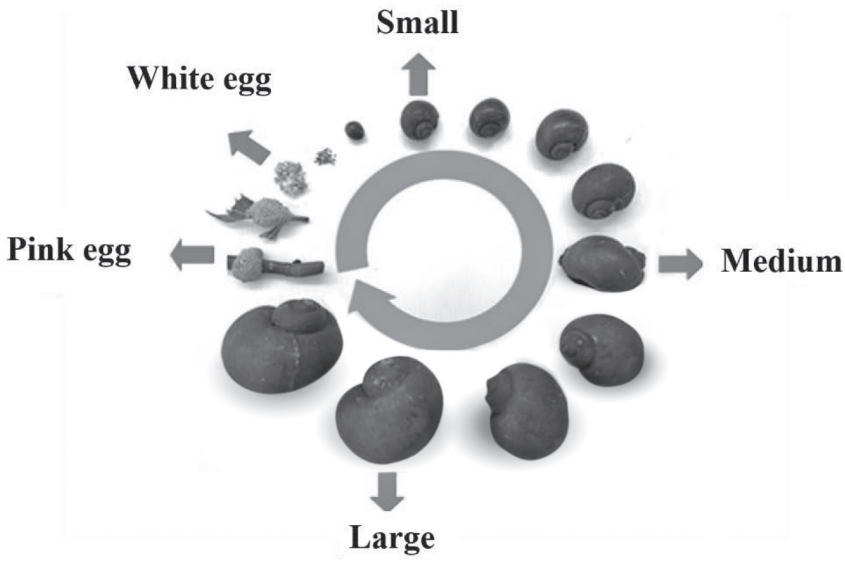


Figure 2 Golden apple snail and its egg collected from Huai−Saneng Reservoir, Surin Province for evaluating AChE expression

Extraction of AChE from Golden Apple Snail and Egg

The extraction protocol was developed based on the method of Thanomsit *et al*. (2017). Both groups of snail egg were cut and mashed with 0.02 M of Tris−HCl (pH 7.2) and 0.01 M of Phenylmethylsulfonyl fluoride (PMSF) in the ration of egg: buffer as 1 g: 1.5 ml. After that, it was centrifuged at 3500 RPM for 1 h and then supernatant was kept in 4°C until AChE expression evaluation was performed.

All of sampled snail was transferred to laboratory and washed. Next, shell characteristic, color and tissue were preliminarily studied and recorded. Then, the snail head was sectioned to extract protein for AChE diagnosis. For one sample, tissue from the part of snail head (10 snails) was mashed and weighed. 1.5 g of mashed tissue w ere filled with buffer as same as the extraction from the egg. Then, they were centrifuged at 3,500 RPM for 1 h and supernatant was kept at 4°C for further analyze AChE expression.

Protein Determination

The protein concentration in both egg and tissue was calculated before applying into gel electrophoresis and Western Blot analysis. Firstly, supernatant was thaw in room temperature. Then, it was diluted with phosphate buffer of pH 7.2 in the ratio of 1:50. Next, the sample was placed in 96 well plate: 10 µl per well for duplication. The BSA protein standard (1.4 mg/ml from Bio−Rad company) was diluted as same as the procedure applied to sample to reach the concentrations of 0.7, 0.35, 0.175 and 0.0875 mg/ml. Then, it was added with 200 µl Bradford Dye Reagent in every hole and placed in room temperature for 5 min. Next, the 96 well plate was measured for absorbance using microplate reader with wavelength at 595 nm. The standard curve was developed from absorbance level against each concentration of BSA protein standard. The X axis was protein concentration (mg/ml) and Y axis was absorbance level. The relation of protein concentration and absorbance was expressed in linear regression equation ($y = ax + b$) and apply to prepare sample for analyze AChE expression in egg and tissue.

12.5% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (Sds−Page)

The ingredients of 12.5% separating gel and 4% stacking gel were prepared. After gel was polymerized it was filled in electrophoresis set. Then, electrode buffer was filled. The mixture of sample and sample buffer were prepared and boiled in water for 5 min. Next, 10 µl (60 µg of protein) was loaded in each channel. A 120 V of electricity was applied until bromophenol blue band reach to gel bottom. The gel was removed and then dyed with Coomassie Brilliant Blue R−250 for 2 h. Next, it was washed by Destaining solution I and II until protein band being seen. The protein size was calculated and compared to molecular weight maker.

Western Blot Analysis

After protein was separated by SDS−PAGE technique, it was transferred from gel onto nitrocellulose paper. In brief, the paper was cut to fit gel plate. The nitrocellulose paper and filter paper were soaked in Semi−Dry Transfer Buffer. Then, nitrocellulose paper was articulated to gel plate and covered with filter paper, and bubble was displaced. Next, it was placed on Trans−Blots SD Semi−Dry Electrophoretic Transfer Cell. Nitrocellulose paper was in lower side. A 15 V of electricity was applied for 15 min. Then, nitrocellulose paper was removed and soaked in 5% blotto dissolved in PBS of pH 7.2 for 1 h. After that, it was washed with PBS/0.5%Tween 20 for 5 min three times. Next, the paper was incubated with polyclonal antibody dilution 1:50 overnight and then excessive antibody was washed with PBS/0.5% Tween20 for 5 mins three times. It was then soaked in Goat Anti−Rabbit Conjugated Peroxidase (GAR−HRP dilution 1:1,000) for 1 h and excessive antibody was washed with PBS/0.5% Tween 20 for 5 min three times. Finally, it was soaked in substrate solution (0.03% DAB, 0.06% H_2O_2, 0.05% $CoCl_2$ in PBS) to develop protein color. The paper was then washed with distilled water and filled with clorox to inhibit substrate reaction. The dark brown color indicates positive result.

Study in Organophosphate and Carbamate Contamination in Water from Huai−Saneng Reservoir, Surin Province by using GT−Pesticide Test Kit

For evaluation of organophosphate and carbamate contamination in water, we used GT−Pesticide Test Kit purchased from Higher Enterprises Co., Ltd. Water sample was collected in the same site where golden apple snail and its egg were also collected. There were 5 sampling stations and the collection was performed in 2 months in June and July 2017. The contamination was compared to negative control and positive control (standard agent). In brief, water sample was placed in test tube of 0.25 ml. Then, it was incubated in a tray filled with heated water of 32−36°C. Next, a 0.5 ml of solvent was filled in each tube and left for 10 min. The results were indicated based on its color comparing to negative control and positive control (detection limit at 0.05 mg/kg as trichlofon).

Statistical Analysis

Statistical analysis was performed to study the difference of protein amount extracted from golden apple snail and its egg. The achieved data was presented in the form of average. The difference of data during sampling month was analyzed by using Indipendence sample T−test. The difference of protein amount among each sampling station and snail size was analyzed by one−way analysis of variance (ANOVA) with Duncan's multiple range test using SPSS 22.0 program.

RESULTS AND DISCUSSION

The land use transformation from local village to urban or crowded domestic area, and intensive agro−chemicals application for increasing yield of rice and other crops create adverse effects to waters and organisms. The important farming areas in Surin province are located nearby Huai−Saneng Reservoir. Moreover, there are many aquatic farms or hatchery in this reservoir, thus contaminants may affect to them.

The golden apple snail presently spread in Thailand is not native species. It was imported from South America around ten years ago for commercial aquatic farming. The snail products were exported to other countries. Later, the demand of snail flesh decreased making the farmers abandoned their farms and many snails creped to the environment. After that, the snail spreads into many parts of the country.

In present, AChE is very important in toxicological testing because it indicates the direct adverse effect onto aquatic organism thus it is an appropriate tool for bio−monitoring to evaluate water quality. Moreover, we can apply in water quality management. Walker *et al*. (2006) indicated that AChE was an appropriate specific biomarker in freshwater fish for monitoring pesticide contamination. In 2009, Somnuek *et al*. (2009) reported that Chlorpyrifos and Carbaryl affected onto the expression of AChE gene in hybrid catfish. The expression in exposed group was higher than that in the control after 24 h of exposure. And, the highest expression was found in the concentration of 43 µM for chlorpyrifos and 119 uM for Carbaryl. And, Jinju *et al*. (2013) also evaluated the effect of Round up in the liver of *Carassius aurata* and found that they were induced to produce higher AChE after exposure for 3 days in the concentration of 32 µg/L compared to the control group. However, the exposure concentration and study method must be considered to study the effect of exposure (Walker *et al*., 2006) for assessing AChE expression. In conclusion, AChE can be used as bio−indicator for pesticide contamination. In toxicological and environmental quality assessment, it can be performed in both fish and shellfish. However, the knowledge on applying gastropod as bio−indicator in Thailand was less. For example, Thanomsit *et al*. (2017) and Chitmanat *et al*. (2008) reported AChE expression in river snail, pond snail and golden apple snail were used for further evaluating pesticide exposure. The achieved results could be used as fundamental data to reduce health risk the consumers and management the water quality.

There are plenty of golden apple snails found in Huai−Saneng Reservior. In this study, we aimed to apply AChE as specific bio−indicator for pesticide exposure (Walker *et al*., 2006) in egg and tissue of golden apple snail. We collected snail's egg in 2 stages: pink and white colored. And, the snail was separated in 3 sizes: small, medium, and large. The experimental period was in rice farming period in the rainy season (June to July 2017). We collected the snails from 5 stations around the reservoir.

The snail's egg collected was in early hatching stage. Basically, there is no report in AChE extraction from snail's egg for indicating pesticide exposure. We found only the study of Heras *et al*. (2007) which reported founding of over identified 59 proteins in the perivivellin fluid in the eggs of *P. canaliculata*. Most of that protein has not been isolated, characterized or identified its function. After their molecular structures were studied, it shows that they function besides storage proteins, and defenses against predation and abiotic factors. They are a mixed function composed of neurotoxic, antinutritive and anti−digestive with providing bright and conspicuous color (aposematic signal) to the eggs.

Thus, this is the first report in Thailand for extracting AChE from egg of golden apple snail and further study cross−reaction with commercial polyclonal antibody specific to AChE by using Western blot analysis. The snail samples collected from 5 stations were normal appearance. Their shell was dark brown, no cracks or any corrosion (Figure 3A). After its lid was opened to check the tissue, the head was normal (Figure 3B). The tissue of snail from stations 1, 2 and 3 was normal without decayed and black; however, the color of snail from stations 4 and 5 was pale (Figure 3D).

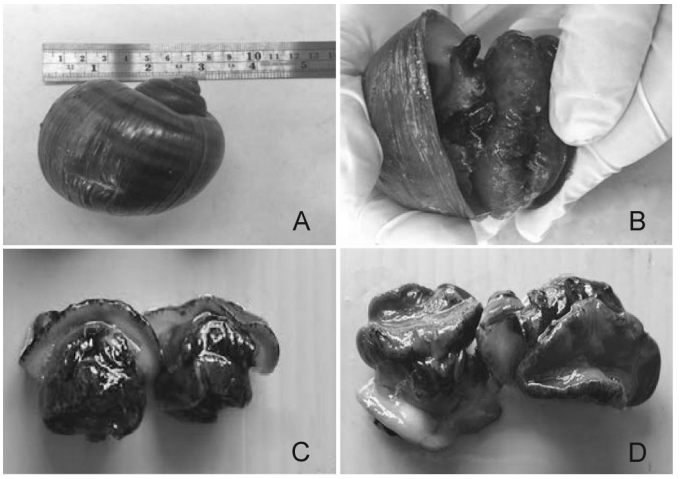


Figure 3 Outside and inside appearance of golden apple snail from Huai−Saneng Reservoir, Surin Province: (A) shell color was dark brown, (B) tissue from head was dark, (C) normal tissue was dark, (D) pale color

Protein concentrations in snail's egg and tissue were evaluated in June and July. We found that protein concentration of egg in post−fertilization stage (pink color) was higher than that in pre−hatching stage (white color) in every station (Figure 4). This might be caused by Lipoprotein such as Perivitellin 2 and Ovorubin which are important protein found in egg cell in early spawning stage (Dreon *et al*., 2003). Perivitellin 2 and Ovorubin are protein being high stability and resist to degradation and pH alteration (4−10) (Cadierno *et al*., 2017). However, the results of protein determination performed in extracts from head of snail in small, medium and large size indicated that snail size had no influence to extracted protein content.

There was no difference in protein amount between 2 sampling months. For sampling stations, station 3 showed the significantly highest protein amount ($P < 0.05$). For the snail sizes, we found that protein amount in the medium and the large were significantly higher than that in the small ($P < 0.05$). Moreover, the significantly highest protein amount ($P < 0.05$) was found in the large snail (station 2) and the medium snail (station 3) collected in June and the large snail (station 3) collected in July (Figure 5).

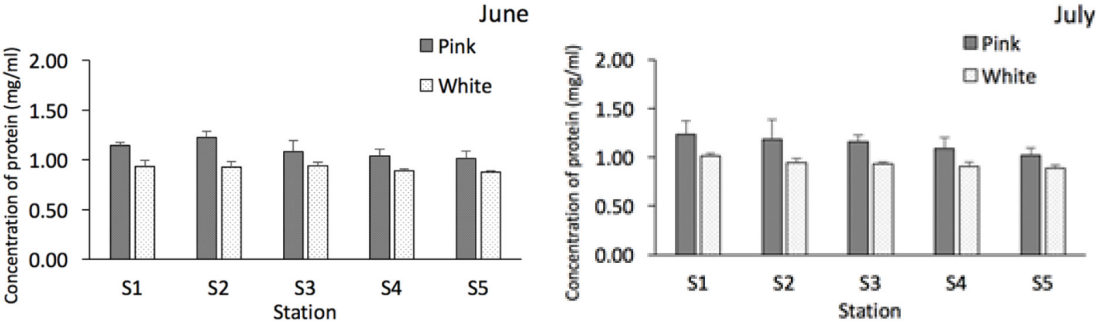


Figure 4 Protein concentrations of snail's egg in pink stage (post−fertilization egg) and white stage (pre−hatching egg) in June and July, 2017

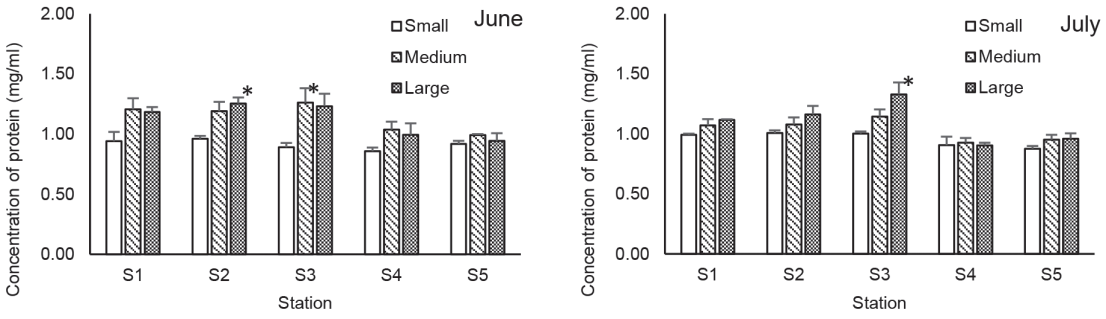


Figure 5 Concentrations of protein extracted from head tissue of golden apple snail in June and July, 2017; group with asterisks expressing significantly higher protein content than other groups ($P < 0.05$)

After studying protein form of snail's egg in both stages, we found the difference as follows. In post−fertilization egg, AChE was dark thick band with the weight of 71 kDa and 66 kDa, respectively (Figure 6A). For the pre−hatching of egg, we found only one AChE protein band (1 isoform) (Figure 6B). The expression of AChE we found was in agreement with the study of Thanomsit *et al*. (2017) that study on AChE expression in river snail and golden apple snail using SDS−PAGE and Western blot technique. They found that AChE protein weight was 71 kDa. After compared AChE expression in all stations, we found that the expression in snail's egg from stations 4 and 5 was lower than that of other stations noted by pale color of protein band. This finding may be caused by continuous exposure of pesticide. In stations 4 and 5, the farmer plants rice twice a year thus they also frequently apply pesticides that this is confirmed by high concentrations of organophosphate and glyphosate as shown in Table 1.

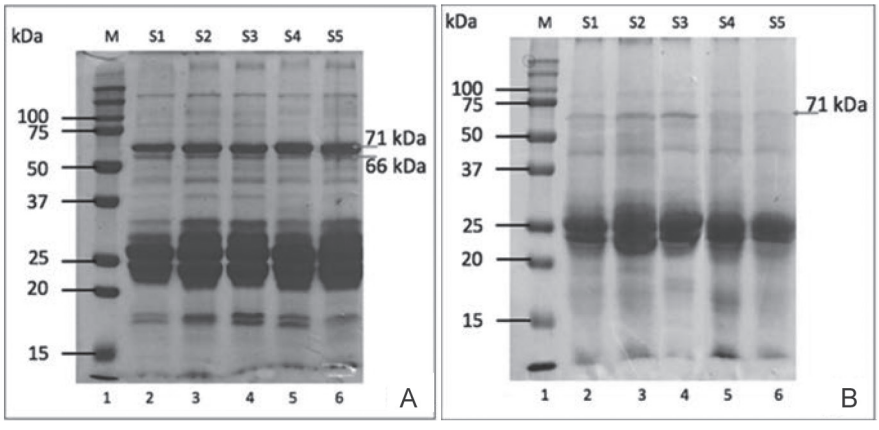


Figure 6 Pattern of SDS−PAGE expressed protein band in golden apple snail's egg: (A) egg in post−fertilization stage from 5 stations, (B) egg in pre−hatching stage from 5 stations; where M as molecular weight marker and S as station

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) was used to study the form of protein found in snail tissue as same as apply in snail's egg. We did not find any differences in protein extracted from small (Figure 7A), medium (Figure 7B), and large (Figure 7C) snail. However, AChE expression in the stations 1, 2 and 3 were higher than that in stations 4 and 5. The weight of AChE protein found in three snail sizes was 71 kDa. Our findings were different from the result of Ma *et al*. (2011) that they purified and studied some characteristics of AChE in *Pardosa astrigena* L Koch by using 2 steps of chromatography: DEAE–52 column and Superdex 200 column (the sample was coagulated by ammonium sulfate before column separation). They found that AChE protein weight was 66.35 kDa. The difference might be caused by technique used.

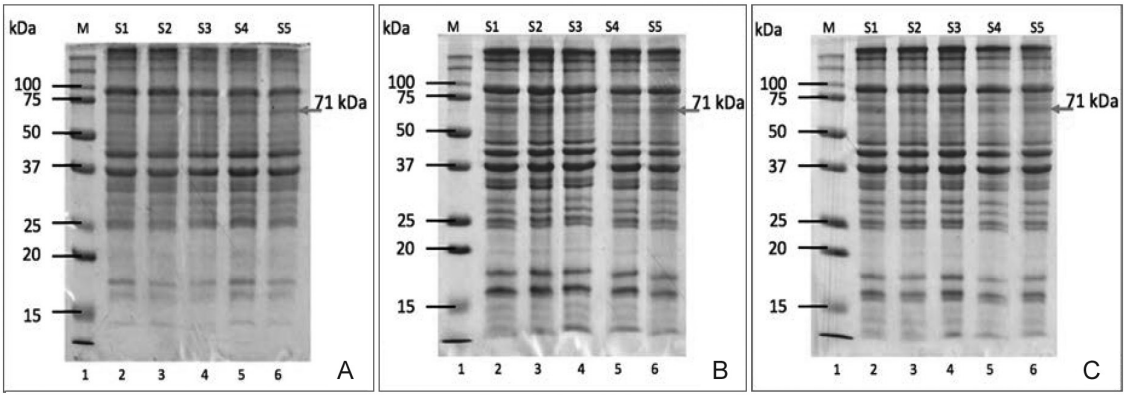


Figure 7 Pattern of protein form extracted from head tissue of golden apple snail by using 12.5% SDS−PAGE: (A) small, (B) medium, (C) large from 5 stations; where M as Molecular weight marker and S as Station

In addition to assess the activity of the AChE enzyme, immunological techniques are another tool for evaluating the expression of AChE. AChE activity in *Schistosoma japonicum* that after testing by Western blot technique using rabbit anti–Sj AChE antibody, the extracted AChE found was 76 kDa. Moreover, quantitative analysis by using Fluorescence–based enzyme assays indicated that highest AChE activity found on the surface skin of matured *S. japnicam*. However, the size of AChE was different from this study that found 2 isoforms: 71 kDa and 66 kDa in egg and tissue of golden apple snail, respectively (Figure 8 and 9).

Chitmanat *et al*. (2008) studied on organophosphate and carbamate pesticide contamination in Ping River in Chaingmai area by using AChE in river snail (*Sinotalia ingallsiana*) as biomarker. This area is classified as intensive agriculture. They found that AChE activity from studied area was lower than that from the control group in every season. They reasoned that a decreasing in AChE activity might be caused by intensive agriculture. These findings are different with our results which found that AChE expression in both snail's egg and the tissue (all three sizes) from stations 1, 2, and 3 was higher than that in the control (Figure 8). However, the expression in stations 4 and 5 were lower than that in the control. This difference might be caused by sample collection performed in rice farming period with low pesticide application. In addition, the intensive of agriculture or residential density in stations 1, 2, and 3 were lower than that in stations 4 and 5. These may induce AChE production in a short time after

exposure. For the lower AChE expression found in stations 4 and 5, it may be explained that more pesticide was applied in this area all year round.

After interviewed the farmer using questionnaire, we found that they applied pesticide in all rice farming period; however, the pesticide used was legally registered (data not shown). This result is in agreement with the study of Smina *et al*. (2016) that evaluated the effect of Thaiamethoxam exposure in *Helix aspersa*. They found that AChE inhibition was depended on the concentrations exposed. AChE was lowest in the case of highest exposure. Moreover, Chitmanat *et al*. (2008) suggested that AChE was a good early warning indicator for pesticide contamination in an aquatic environment. However, it should be more studied in other biochemical markers such as histopathology effects, growth, reproduction and survival of aquatic organism. In addition, the season also effects on the results especially the rainy season which is normal farming period. Singh (2014) studied on AChE activities in nervous system of snail (*Lymmaea acuminata*) for applying as bio−indicator in Ramgarh Lake, Gorakhpur, UP, India. They found that season affected on AChE activities. Furthermore, Singh *et al*. (2008) studied on the toxicity of carbaryl in *L. acuminate* during 2006−2007 (every month). The LC_{50} values (24 h) determined in an out−door study with estimation of levels of abiotic factors including temperature, pH, dissolved oxygen, carbon dioxide and electrical conductivity in dechlorinated tap and pond water. They found the highest inhibition of 55.26% of AChE activity in August. For our study, we performed in only 2 months June and July because it is planting period of rice, corn and tomato etc. After contamination of organochlorine and carbamate insecticide was evaluated by using GT−Pesticide Test Kit, we found the contamination in every station and time including June and July. The highest contamination was in stations 4 and 5. By interviewing, we found that there was in and off season (with irrigation) paddy field in stations 4 and 5 while it was only seasonal rice farming in stations 1, 2 and 3 resulting in lower contamination of organophosphate and glyphosate.

Moreover, it was found that development stage, egg size, sampling station and exposure time influenced on AChE expression in snail egg and its tissue on June and July as shown in Table 1. In 2018, Nongnutch (2018) indicated that AChE enzyme activity could be found in egg of golden apple snail aged 1 day. As generally known, nervous system and brain are target organs of pesticides. Besides, they were also found in other organs. In 2010, Modesto and Martinez (2010) studied the effect of Roundup (glyphosate) in the concentration of 10 mg/L on brain and muscle of *Prochilodus lineatus* at 6, 24 and 96 h after exposure. They found that the AChE in brain and muscle of exposed fish was higher than that of control group after 6 h. For enzyme activity, the activity in exposed fish brain was still higher than that in the control group after 24 h exposure which contrasted to the activity in muscle. In the muscle, the activity significantly decreased ($P > 0.05$). Besides, enzyme activity in both brain and muscle of exposed fish was lower than that of the control group after 96 h exposure.

Additionally, Anandhan *et al*. (2012) studied the function of AChE in 2 species of freshwater bone fish: *Channa striatus* and *Oreochromis mossambicus* in India. It was found that AChE activity was different in each studied tissue: brain > muscle > gill > liver. In this research, we studied the protein in snail egg because it has the risk of pesticide exposure. After the protein form was studied by using SDS−PAGE, we clearly found the protein form with the sizes of 71 kDa and 67 kDa. Our findings are in agreement with the study of Ma *et al.* (2011) that studied purification and some characteristics of AChE in *Pardosa astrigena* L.Koch by using column chromatography technique. They found that AChE had molecular size of 66.35 kDa, which is similar to the size of AChE found in this study.

However, quantitative assessment such as ELISA technique should be performed for clear confirmation. Based in our findings, we concluded that acetyl cholinesterase (AChE) in the nervous tissue can be applied as sensitive bio−indicator for abiotic environmental factors such as pesticide contamination.

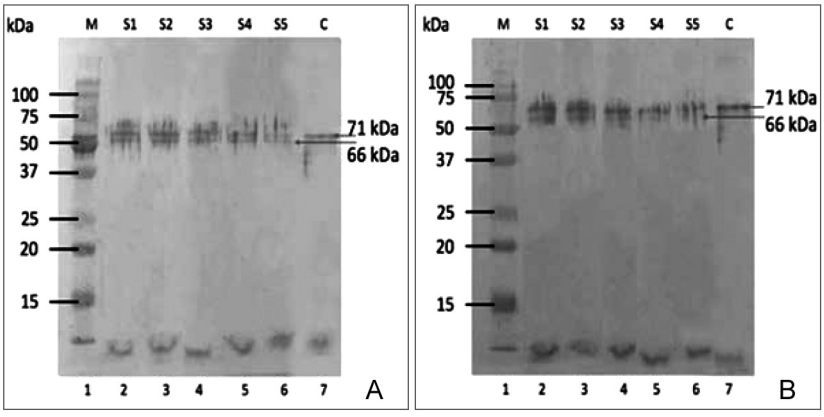


Figure 8 Pattern of Western blot analysis expressing AChE activity in pink stage: (A) and white stage of snail's egg, (B) from 5 stations by using commercial polyclonal antibody dilution 1:200. The amount of AChE tested was 80 µg/lane where M as Molecular weight marker, S as Station, and C as control (purified AChE from hybrid catfish)

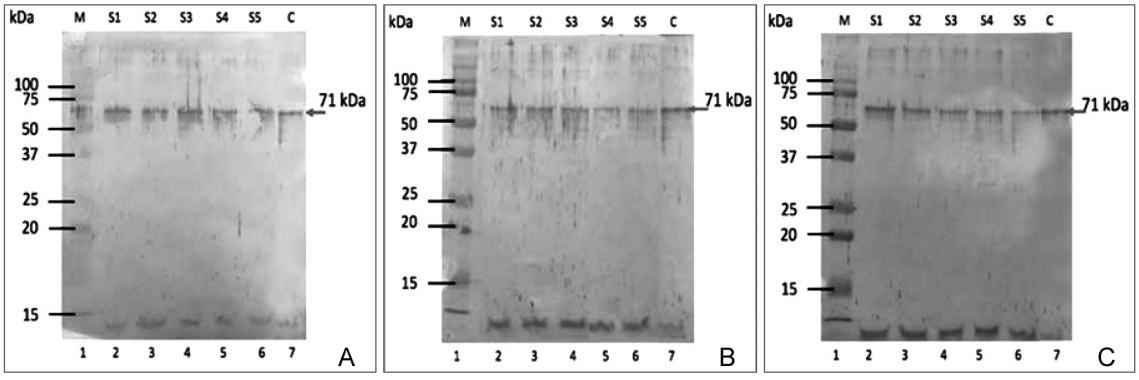
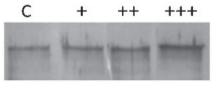


Figure 9 Pattern of Western blot analysis expressing AChE activity in small (7A), medium (7B) and large (7C) golden apple snail from 5 stations by using commercial polyclonal antibody dilution 1:200. The amount of AChE tested was 80 µg where M as Molecular weight marker, S as Station, and C as control (purified AChE from hybrid catfish)

Table 1 Western blot analysis of AChE expression in snail's egg and its tissue of golden apple snail and results of organophosphate and carbamate contamination tested by GT−Pesticide test kit

Type of sample	Size and stage	Results									
		June 2017 (Station)					July 2017 (Station)				
		1	2	3	4	5	1	2	3	4	5
*Egg	pink	+++	++	+++	++	+	+++	++	+++	+	+
	white	++	++	++	+	+	+++	++	++	+	+
*Tissue of golden apple snail	small	+++	+++	+++	++	+	+++	++	+++	+	+
	medium	++	++	++	+	+	++	++	++	+	+
	large	+++	+++	+++	+	+	+++	++	++	++	+
**Water	surface water	+	+	+	++	++	+	+	+	++	++

Remark: * Tested by Western blot analysis, ** Tested evaluated by GT−Pesticide Test Kit,



+ low expression, ++ medium expression, +++ high expression

Furthermore, bio−monitoring application based on biochemical and immunological responses in the exposed organism can be used in setting more accurate environmental quality classes with considering the interference effects of other competitive factors. For better understanding, AChE recovery after exposed to organophosphorous and carbamate pesticides should be further studied. In addition, the appropriate endpoints and organisms applying in risk assessment should be also investigated. Moreover, the organisms living in agricultural areas which generally exposed to mixed pesticides have been a difficult task to be managed and regulated because our knowledge was mostly based on single substance toxicity. The possible synergistic and/or antagonistic effect among various pesticides must be further studied and identified.

CONCLUSION

In agricultural area such as farming fields nearby Huai−Saneng Reservior, the application of insecticide in many proposes can cause contamination and accumulation in soil, water and living organisms. The assessment of biological effect such as AChE expression (specific biomarker) can give exactly occurred effect in the organisms. In this study, AChE expression was used in assessing insecticide exposure. There was an evidence to show that AChE could be efficiently apply in both egg and tissue of golden apple snail which performed in farming period in June and July, 2017. In addition, we found that the contamination of organophosphate and carbamate in water was consistent with AChE expression in every station. In fertilization egg (pink stage), it found two isoforms: 71 and 66

kDa while it was only one band in post fertilization egg (white stage): 71 kDa with lower protein content. Thus, golden apple snail has a high potential to be applied as bio−indicator for pesticide contamination for further planning of environmental management.

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