Induction and One-Step Purification of Acetylcholinesterase (AChE) from the Brain of Hybrid catfish after Exposed to Glyphosate

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Received: 20 May 2020; Revised: 21 July 2020; Accepted: 30 July 2020

Abstract

Acetylcholinesterase (AChE) is classified as a specific biomarker for herbicide exposure in aquatic organisms. In this study, we investigated an AChE induction in the brain of juvenile and adult hybrid catfish after exposoure to glyphosate in sub-lethal concentration for quantifying the highest yield of AChE. Moreover, we separated and purified AChE by using a one-step column chromatography technique, which could be an alternative method. Based on the results, the appropriate glyphosate concentration for induction of AChE in juvenile catfish hybrids was 0.2 ml/ L while that in adult was 0.75 ml/L. And, hydroxyapatite was applied as a media for separation and 0.2 M phosphate buffer was applied for protein elution. After comparing the suitability of hybrid catfish for extracting AChE, it was found that adult hybrid catfish was more suitable than the juvenile. The molecular weight of separated AChE was 71 kDa when studied by using SDS-PAGE and Western blot techniques. For our results, the columnar chromatography technique with hydroxyapatite was likely to be used to purify AChE from the brain of a hybrid catfish in only one step. This is a useful alternative method in separating AChE in order to apply as an antigen to detect pesticide exposure. And, it can be further applied to production of polyclonal antibodies and monoclonal antibodies specific to AChE for monitoring of pesticide exposure.

Keywords: acetylcholinesterase, glyphosate, antibody techniques, toxicity testing

Introduction

Presently, an increase in consumer's needs results in an imported volume of pesticides for applying in agricultural areas. In Thailand, the pesticide is applied as an efficient tool for protecting agricultural products and improving their qualities in both size and appearance (Health System Research Institute, 2005).

Glyphosate is an herbicide, widely used in Thailand. It is in a top ranking of imported agrochemicals though it was banned in many countries (Praneetvatakul, Schreinemachers, Pananurak, & Tipraqsa, 2013). It was synthesized in 1950 by Dr. Henri Martin who is Swiss chemist. Originally, its ability in eliminating weed was still not known and afterward the Monsanto's scientist developed and applied it as an herbicide (Dill et al., 2010).

Acetylcholinesterase (AChE) will be inhibited because of binding with glyphosate at serine hydroxyl group. After that, acetylcholine is accumulated and then nerve impulse is strongly stimulated causing anxiety and uncontrollable movement. In the case of exposure to excessive concentration, it can cause organisms to die. From the importance mentioned above, AChE is likely to be applied as an indicator of contamination of glyphosate, which is an herbicide extensively found in aquatic environments and organisms.

In aquatic ecosystem, glyphosate will inhibit AChE activity in the organisms. After being taken up into the body, glyphosate binds with cholinesterase enzyme making the enzyme is not capable of degrading acetylene with its function as nerve signals between neuroreceptor and muscle. The undegraded acetylcholine is accumulated, making nerve signals continuously; then, the muscle may be contracted, paralyzed and death. Because of the importance of cholinesterase enzyme onto physiological appearance of fish thus it can be applied as an indicator of aquatic toxicity (Menėndez- Helman, Ferrey, Santos Afonso, & Salibian, 2012). Moreover, Gruber and Munn (1998) suggested that the level of AChE can be used as a bio-monitor especially in a risk area for agrochemical contamination. The level of AChE in both brain and blood of exposed fish was high thus it is appropriate to be a useful tool for evaluating the effect. In addition, Walker, Hopkin and Peakall (2006) indicated that AChE has been classified as a specific biomarker for insecticide and herbicide exposure in aquatic organisms thus it is very important for environmental toxicological study.

It was reported that AChE could be separated and purified by many methods, each with different procedures. For examples, Akman, Turkoglu, and Celik (2009) studied the purification and characterization of AChE from liver and brain of *C.tarichii* fish P.1811, and found that AChE can be separated and purified by using column chromatography techniques. The separated AChE values extracted from brain and liver were 142 times and 344 times when compared to the crude extraction. The appropriate pH values for the purification of AChE in the brain and liver were 7.5 and 8.5 respectively. And, Ma, Zhang, and Li (2011) investigated the purification and characterization of AChE from *Pardosa astrigera* L. Koch and found that AChE could be purified by using two chromatography columns: DEAE-52 and superdex 200. After studying by using polyacrylamide gel electrophoresis technique, it was found that the isolated AChE had a molecular weight of 66.35 kDa.

The measurement of AChE level can be performed by using many methods such as direct measurement by using spectrophotometer or histological technique based on antigen-antibody interaction principle. The antigen-antibody recognition technique is simple, precise, and inexpensive. However, an application of this technique to measure AChE level is quite difficult because there are many forms and molecular weights of AChE enzyme. Moreover, a commercial antibody mostly used is imported and not specific to economic fish in Thailand. For eliminating limitation mentioned above, this study was aimed to extract and purify AChE enzyme for further use as antigen in polyclonal production specific to AChE in hybrid catfish after exposed to glyphosate and prepared AChE as antigen for screening monoclonal antibody production.

Methodology

Toxicity and responsive testing of hybrid catfish after exposed to glyphosate

The juvenile and adult hybrid catfish (n=10) were placed in a tank filled with 1-10 mg/L for 24-96 h for three replicates. The glyphosate used in this study was in commercial form. The alteration and AChE

induction in fish brain was monitored in different concentrations and exposure times. Then, the cumulative mortality and LC_{10}, LC_{50}, and LC_{90} were calculated using Minitab® 17 software (entitlement i.d.: 2ec6-9b37-1508-0264-2c55-c33).

Extraction and purification of AChE on hybrid catfish

The brain of juvenile and adult hybrid catfish was taken and placed in phosphate buffer (pH 7.2) containing PMSF 0.1 M. The ration of brain and muscle for extraction was 1 g of tissue to 1.5 ml of buffer. Next, tissue was extracted by using homogenizer and then was centrifuged at 5,000 rpm. Finally, the supernatant was kept for further measuring AChE.

AChE was purified by column chromatography technique. The applied protein (extracted from fish brain) was eluted by 0.2, 0.4 and 1.2 M of Sodium phosphate buffer (pH 7.0). Every 1.5 ml eluted fraction (120 fractions) was kept to measure absorbance at 280 nm. The amount of AChE in each fraction was calculated compared to standard solution. And, the molecular weight of AChE was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis technique (SDS-PAGE).

Measurement of total protein

The protein extracted from fish brain was diluted by phosphate buffer saline, PBS (pH 7.4). Next, it was placed in a 96 well plate (100 μL), and then 200 μL of dye reagent concentrate (Bio Rad protein assay) was added. Finally, it was measured for absorbance at 595 nm and calculated for protein amount by comparing to standard curve of Bovine serum albumin (BSA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique (SDS-PAGE)

Acrylamide with the concentration in separating gel as 12% was prepared. Next, 4% stacking gel was prepared by pouring the water onto the comb and stacking gel was then filled. After that, it was left for 30 min for settling. The AChE was mixed with 2X treatment buffer at the ratio of 1:1 (100 μl/100 μl) and boiled in water for 3 min. The sample was filled in gel slit by 10 μg/lane. Next, 120 volts of electric current was applied to make specimen, for moving down until reaching to the bottom of the gel and then stopped. A part of finished gel was taken to be stained by Coomassie Brilliant-blue R-250 for further comparing to protein standard.

Specificity of AChE by using commercial polyclonal antibody

The protein was transferred from gel sheet onto nitrocellulose paper using semi dry-transblot apparatus. Then, it was soaked in 0.5% blotto letting the milk protein bind to rest of nitrocellulose membrane for 1 h at room temperature. Next, it was incubated in antibody specific to AChE for 3 h. The antibody used was diluted in 0.5% blotto at ration at 1:50. Then, it was washed by 0.5% blotto for 10 min for 3 times. It was further incubated in goat-anti rabbit-HRP diluted with 5% blotto at the ration of 1:1000 for 3 h in room temperature and then washed with 0.5% blotto for 5 min three times. Next, it was soaked in substrate solution (0.03% diaminobenzidine (DAB) 0.006%, H_2O_2 and 0.05% $CoCl_2$ in PBS) for 5 min. The dark band indicates AChE was presented.

Results

Toxicity testing, cumulative mortality rate, and LC_{10}, LC_{50} and LC_{90} of hybrid catfish exposed to glyphosate

Toxicity testing of glyphosate on juvenile hybrid catfish

In evaluating glyphosate toxicity onto juvenile hybrid catfish, we applied glyphosate in 7 different concentration levels: 0, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 ml/L in varying exposure times of 24, 48, 72 and 96 h, respectively. The results indicated that mortality percentage was increased with an increase in glyphosate concentration. We firstly found fish death at 24 h, which reached the highest number at 96 h. The highest cumulative mortality rate at 96 h is shown in Figure 1.

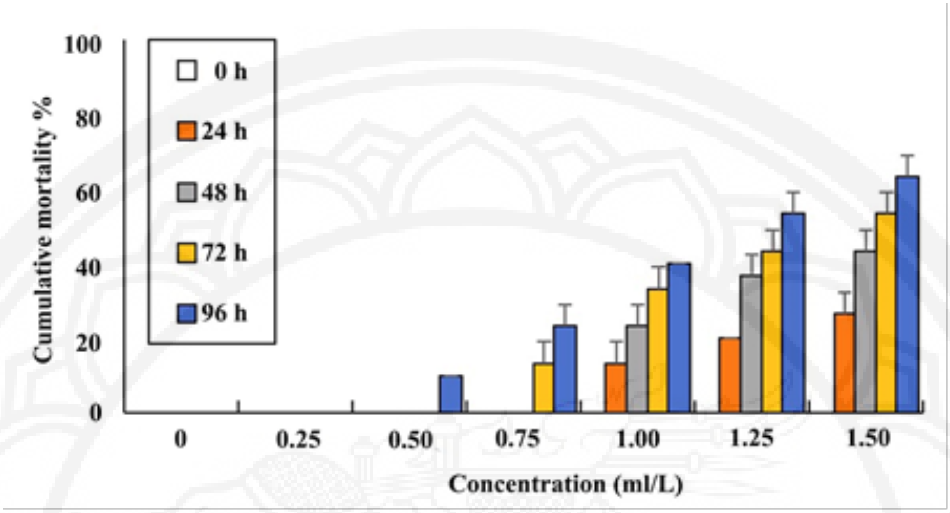

Figure 1 Glyphosate concentration causing mortality in juvenile hybrid catfish

Toxicity testing on adult hybrid catfish

Glyphosate toxicity was performed in the adult hybrid catfish at 6 different concentrations of 0, 0.75, 1.0, 1.25, 1.5 and 2.0 ml/L in exposure times of 24, 48, 72 and 96h, respectively, the results indicated that the mortality percentage was increased with an increasing in glyphosate concentration. The fish death was firstly noticed at 24 h, which reached the highest number at 96 h. The cumulative mortality was at 96 h as shown in Figure 2.

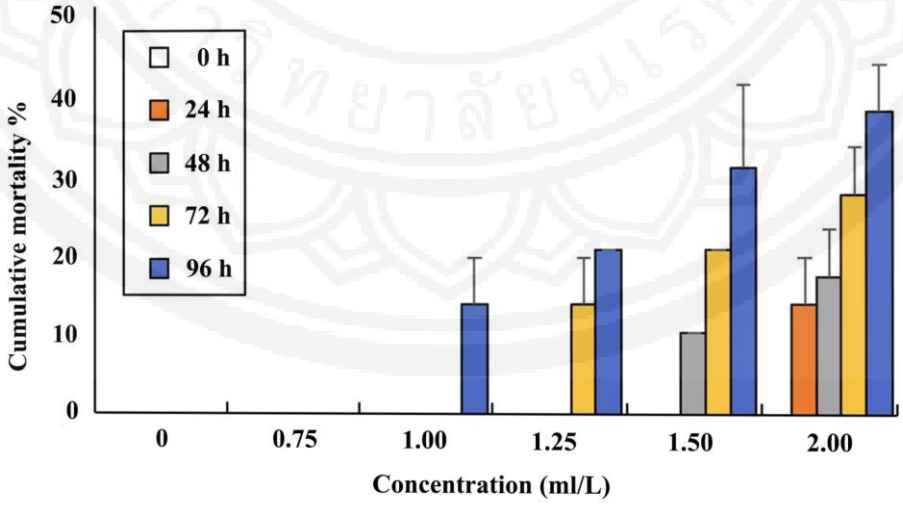

Figure 2 Glyphosate concentration causing mortality in adult hybrid catfish

The LC_{10}, LC_{50} and LC_{90} values were tested and indicated using Probit analysis in both juvenile and adult hybrid catfish. The results are shown in Table 1.

Table 1 Toxicity level of glyphosate in juvenile and adult hybrid catfish

Time	Juvenile			Adult		
	LC_{10} (95% confidence)	LC_{50} (95% confidence)	LC_{90} (95% confidence)	LC_{10} (95% confidence)	LC_{50} (95% confidence)	LC_{90} (95% confidence)
24 h	0.869 (0.707-0.910)	2.508 (2.163-3.219)	7.236 (5.023-13.493)	ND	ND	ND
48 h	0.657 (0.554-0.737)	1.661 (1.567-1.804)	4.194 (3.402-5.757)	1.5 (1.284-1.616)	4.827 (3.525-10.878)	15.531 (7.930-89.35)
72 h	0.639 (0.597-0.677)	1.388 (1.346-1.437)	3.011 (2.740-3.373)	1.017 (0.856-1.129)	3.659 (3.055-4.971)	13.167 (8.436-28.293)
96 h	0.508 (0.479-0.534)	1.192 (1.160-1.227)	2.798 (2.603-3.040)	0.831 (0.747-0.901)	2.598 (2.385-2.909)	8.119 (5.447-11.091)

ND: Not detected

Acetylcholinesterase purification by column chromatography technique for using as antigen for polyclonal antibody production

In this research, AChE was purified using a column eluted with phosphate buffer in 3 different concentrations of 0.2, 0.4 and 1.2 M, respectively. The eluted protein being kept was 120 fractions (1.5 ml/fraction). In performing, we kept 40 fractions in each buffer. After that, the kept protein was applied to measure optical density absorbance (OD) at 280 nm. And, it was also applied to measure protein amount compared to standard protein (BSA). After protein form comparison by SDS-PAGE technique was performed, we found that AChE from brain of juvenile hybrid catfish was lower than that in the adult. Thus, we used adult hybrid catfish in polyclonal antibody production by exposing them to glyphosate in the concentration of 0.75 ml/L for 24 h. After purified by column chromatography, pure AChE achieved was 71 kDa. Then, the fractions of 3, 4 or 5 were applied as antigen in further antibody production (Figure 3).

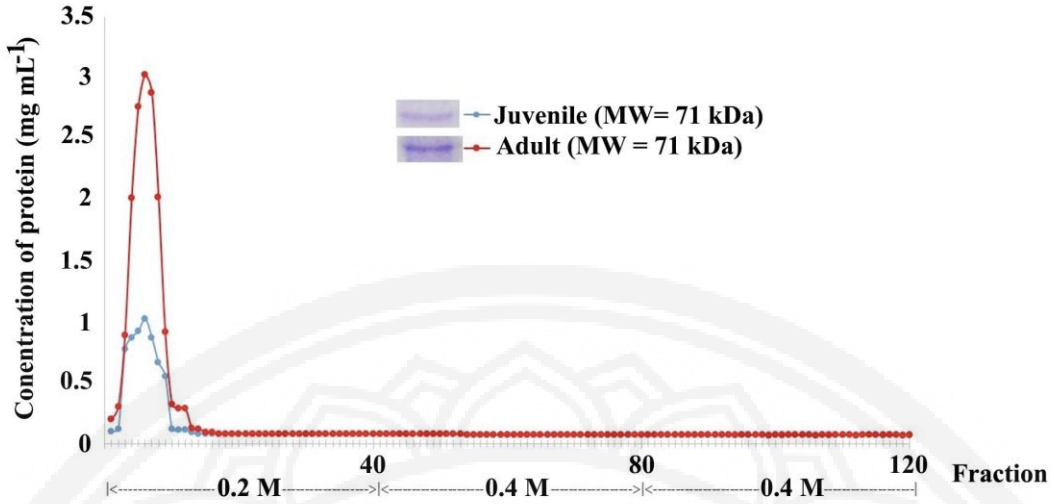

Figure 3 Amount of protein extracted from juvenile and adult hybrid catfish using column chromatography technique

Investigation of protein form in catfish exposed to glyphosate by SDS-PAGE (12 %) technique

Protein form in brain of juvenile catfish after exposed to glyphosate

After juvenile hybrid catfish exposed to glyphosate in the concentrations of 0.2, 0.25, and 5 ml/L for 12 and 24 h, we found that AChE extracted from their brain was 71 kDa compared to standard protein. The appropriate concentration and time were consecutively 0.2 ml/L and 12 h (Figure 4 in lane 3).

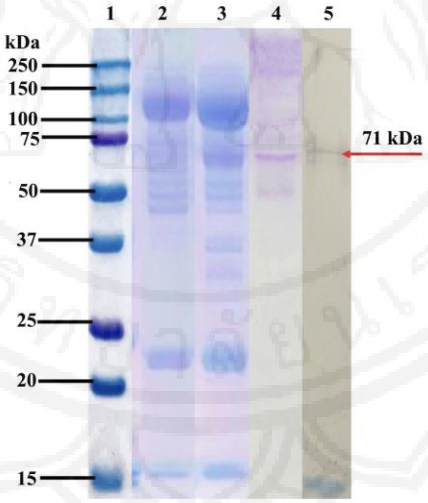

Figure 4 Pattern of protein in brain extracted from juvenile hybrid catfish after exposed to glyphosate comparing to standard protein with appropriate concentration and time of 0.2 ml/L for 12 h

Lane 1: Standard protein (from BioRad Company)

Lane 2: Protein from brain of hybrid catfish in control group

Lane 3: Protein from brain of hybrid catfish exposed to glyphosate in the concentration of 0.2 ml/L for 24 h

Lane 4: Protein from brain of hybrid catfish exposed to glyphosate in the concentration of 0.2 ml/L for 24 h and being purified by column chromatography technique

Lane 5: Protein from brain of hybrid catfish exposed to glyphosate and being tested by Western blot technique using commercial polyclonal antibody having protein band of 71 kDa

Protein form in brain of adult hybrid catfish after exposed to glyphosate

After the protein extracted from brain of adult hybrid catfish exposed to glyphosate in the concentrations of 0.75, 1.0, 1.25 and 1.5 ml/L for 24 h was studied, the results showed that extracted AChE was 71 kDa compared to standard protein. The appropriate concentration and time were respectively 0.75 ml/L and 24 h (Figure 5).

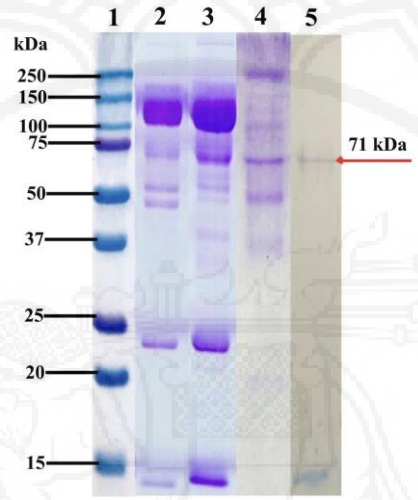

Figure 5 Pattern of protein in brain extracted from adult hybrid catfish after exposed to glyphosate comparing to standard protein with appropriate concentration and time of 0.75 ml/L for 24 h

Lane 1: Standard protein (from BioRad company)

Lane 2: Protein from brain of hybrid catfish in control group

Lane 3: Protein from brain of hybrid catfish exposed to glyphosate in the concentration of 0.75 ml/L for 24 h

Lane 4: Protein from brain of hybrid catfish exposed to glyphosate in the concentration of 0.2 ml/L for 24 h and being purified by column chromatography technique

Lane 5: Protein from brain of hybrid catfish exposed to glyphosate and being tested by Western blot technique using commercial polyclonal antibody having protein band of 71 kDa

Discussion

Presently, AChE is very important in toxicological investigations because it can directly express negative effects on organisms, especially for an application of biological indicator to assess the quality of aquatic environment. And, it can be further used as a guideline in managing water quality. AChE can be used as a biological indicator for pesticide contamination in aquatic animals because these substances inhibit activity of AChE in hydrolyzing acetylcholine into choline and acetate, thus causing the accumulation of acetylcholine at

the nerves junction and then resulting in nervous system to malfunction (Colović, Krstić, Lazarević-Pašti, Bondžić, & Vasić, 2013).

Currently, extraction and purification of AChE have become an interesting issue because it is used as an indicator of exposure to pesticides. Regardless of the technique used, extraction and quantitative determination will lead to accurate inspection. Many techniques applied to detect the expression of AchE were reported in aquatic animals such as enzyme activity monitoring as shown in the studies of Modesto and Martinez (2010). They studied the effect of Roundup (glyphosate) in concentration of 10 mg/L in the brain and muscles of *Prochilodus lineatus* at 6, 24 and 96 h after exposure. They found that AChE expression in both brain and muscles were higher than that in the control group at 6 h of exposure. After examining the enzyme activity after 24 h, the result indicated that enzyme activity in the brain of exposure group was higher than the control group. In contrast to the enzyme activity in the muscles, they were decreased significantly ($p<0.05$). And when exposed for 96 h, it was found that detected enzyme activity was lower than the control group in both the brain and muscles. Rakhi, Mohsinul Reza, Hossen, and Hossain (2013) studied the changes in tissues and examined AChE activity in the brain of catfish (*Heteropneustes fossilis*) collected from a polluted area of Bangladesh. They found that fish tissues were destroyed in many important organs such as skin, muscles, liver, gill and kidney. The station where found the highest acetylcholinesterase activity was Shitalakkhaya with an average of 130.67 ± 3.51 nmol/min/mg protein.

In this study, the toxicity testing of glyphosate on hybrid catfish, we found that the juvenile was higher susceptible than that in the adult. The LC_{50} values at 24, 48, 72 and 96 h in the juvenile hybrid catfish were 1.68, 1.79, 1.45 and 1.25 ml/L, respectively, while the LC_{50} values at those in the adult were higher. However, we could not assess by Probit analysis because there was no mortality in experimental period (24 h). In adult hybrid catfish, the LC_{50} values at 48, 72 and 96 h were 3.67, 5.42 and 3.38 ml/L, respectively. Our results were different from the study of Jidee, Jitgla, Natpra, Udduang, and Thanomsit (2016) which was not found any death in the concentrations of 1.0, 1.25 and 1.50 ml/L, except at 1.75 ml/L had 10% mortality at 96 h. This might be caused by the difference between fish species and applied substance form. Thus, it could not calculate LC_{10} and LC_{50} of glyphosate in common climbing perch at 96 h. However, in assessing LC_{10} value, at least two points of data were required. Thus, the results only showed the trend of mortality in common climbing perch. Besides, the results of this study were not in agreement with the study of Harayashiki et al. (2013) who reported Guppies fish (*Poecilia vivipara*) which exposed to glyphosate in the concentrations of 1, 30 and 700 μg/L (in commercial form) showing low sperm production and quality. And, DNA alteration and mortality were also noticed. This finding might be caused by the difference in the form of chemical applied and fish species (Walker et al., 2006). In addition, this result is different from the findings of Thanomsit, Wattanakornsiri, and Nanthanawat (2016) who studied the effect of glyphosate on Asian Sea bass after exposed for 24, 48, 72 and 96 h and found that LC_{50} at 24 h was 5.57 ml/L. The LC_{50} values at 48 h, 72 h and 96 h were respectively 3.55, 2.5 and 0.76 ml/L.

AChE is classified as high specific biomarker and applied for indicating the exposure of herbicide (Walker et al., 2006). There are many methods to measure AChE such as molecular technique (Braz - Mota, Sadaus-Henrique, Duarte, Val, & Almeida-Val, 2015). In addition, spectrophotometry can be applied indirect measurement. Moreover, immunological technique based on antigen-antibody specificity is also an alternative

method for applying because it is easy, highly specific, and inexpensive (Prasartkaew, Thanomsit, & Nanthanawat, 2016). Moreover, Thanomsit et al. (2017) and Thanomsit et al. (2018) reported that AChE detected in golden apple snail and pond snail by using western blot technique had molecular weight of 71 kDa. And, polyclonal antibody production and monoclonal antibody are very important in this method. In this study, we aimed to extract and purify AChE from brain of hybrid catfish exposed to glyphosate. The purified AChE was 71 kDa which was similar to findings in the study of You et al. (2016). They studied on AChE in *Schistosoma japonicum* and found that the extracted AChE was 76 kDa and then further examined by Western blot technique using Rabbit anti – AChE antibody. And, our results are in agreement with the study of Ma et al. (2011) who investigated the purification and specific characteristic of AChE from *Pardosa astrigera* L.Koch. They purified AChE using 2 chromatography columns: DEAE-52 and Superdex 200. After polyacrylamide gel electrophoresis applied, we found that molecular weight of extracted AChE was 66.35 kDa. Moreover, Menėndez- Helman et al. (2012) studied the toxicity and effect of glyphosate in *Cnesterodon decemmaculatus*, which the concentrations of glyphosate used were 1, 17.5 and 35 mg/L. The fish was alive along the experimental period in three conditions and AChE enzyme activity was 23-36%. Thus, it could be concluded that AChE can be applied as bio-indicator of glyphosate toxicity in brain. Besides, Glusczak, Santos, Crestani, and Pimental Veieira (2006) studied the effect of glyphosate on the activity of AChE enzyme in bony fish (*Leporinus obtusidens*) exposed to herbicide for 96 h in the concentrations of 0, 3, 6, 10 and 20 mg/L. They found that enzyme activity in brain was significantly higher than that in muscle ($p<0.05$). AChE enzyme activity in brain was 13.8 ± 0.76 μ mol /min/g protein while it was 6.1 ± 1.31 μ mol/min/g protein in the muscle. Based on the results of this study, the activity of AChE enzyme can be used as early warning bio-indicator for herbicide exposure in *Leperinus obtusidens*.

Conclusion and Suggestions

Glyphosate is an herbicide causing toxic effect on both juvenile and adult hybrid catfish. Acetylcholinesterase (AChE) in brain is extracted and purified by column chromatography technique, we found that this technique can be applied to purify AChE for further use as antigen in antibody production. The molecular weight of AChE extracted from both juvenile and adult hybrid catfish was about 71 kDa. After that, cross-reaction testing by using PAb specific to AChE in commercial form was also performed. For improving the efficiency of measurement and antibody production, adult catfish should be used in performing AChE extraction because of its bigger brain and higher protein content affecting antigen preparation.

Acknowledgments

This study was sponsored by the Fisheries Department, Faculty of Agriculture and Technology Rajamangala University of Technology, Isan Surin Campus.

References

Akman, E., Turkoglu, V., & Celik, I. (2009). Purification and characterization of Van Lake Fish (*Chalcalbumus tarichii* P.1811) liver and brain acetylcholinesterase. *Hacettepe journal of biology and chemistry, 37*(4), 331-336.

Braz-Mota, S., Sadaus-Henrique, H., Duarte, R. M., Val, A. L., & Almeida-Val, V. M. F. (2015). Roundup exposure promotes gills and liver impairments, DNA damage and inhibition of brain cholinergic activity in the Amazon teleost fish *Colossoma macroponum*. *Chemosphere*, *135*, 53-60.

Colović, M. B., Krstić, D. Z., Lazarević-Paš ti, T. D., Bondž ić, A. M., & Vasić, V. M. (2013). Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. *Current Neuropharmacology*, *11*, 315-335.

Dill, G. M., Sammons, R. D., Feng, P. C. C., Kohn, F., Kretzmer, K., Mehrsheikh, A., ... Haupfear, E. A. (2010). Glyphosate: Discovery, Development, Applications, and Properties. In V. K. Nandula (Ed.), *Glyphosate Resistance in Crops and Weeds: History, Development, and Management* (pp. 1-34). USA; John Wiley & Sons.

Gruber, S. J., & Munn, M. D. (1998). Organophosphate and carbamate insecticides in agricultural waters and cholinesterase (ChE) inhibition in Common Carp (*Cyprinus carpio*). *Archives of Environmental Contamination and Toxicology*, *35*, 391-396.

Glusczak, L., Santos, M., Crestani, M., & Pimental Veieira, V. (2006). Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic and hematological parameters in Piava (*Leporinus obtusidens*). *Ecotoxicology and Environmental Safety, 65*(2), 237-241.

Harayashiki, C. A. Y., Varela Junior, A. S., Souza Machado, A. A., Costa Cabrera, L., Primel., E. G., Bianchini, A., & Corcini, C. D. (2013). Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water. *Aquatic Toxicology*, *142-143*, 176-184.

Health System Research Institute. (2005). *Knowledge needed for effective decision of Pesticide policy in Thailand.* Nonthaburi, Thailand; Ministry of Public Health.

Jidee, S., Jitgla, U., Natpra, C., Udduang, S., & Thanomsit, C. (2016). Effect of glyphosate on fish behavior and mortality rate in Climbing Perch (*Anabas testudineus*, Bloch). In *Proceedings the 6th Rajamangala Surin Conference 22-23 December 2016* (pp. 193-203). Surin: Rajamangala University of Technology Isan Surin Campus.

Ma, M., Zhang, B., & Li, S. (2011). Purification and partial characterization of acetlcholinesterase from Pardosa astrigera L. Koch. *Journal of Cell and Animal Biology*, *5*(1), 11-16.

Menėndez- Helman, R., Ferrey, G. V., Santos Afonso, M., & Salibian, A. (2012). Glyphosate As an acetylcholinesterase inhibitor in *Cnesterodon decemmaculatus*. *Bulletin of Environmental Contamination and Toxicology*, *88*, 6-9.

Modesto, K. A., & Martinez, C. B. R. (2010). Effects of Roundup Transorb on fish: Hematology, antioxidant defenses and acetylcholinesterase activity. *Chemosphere*, *81*, 781-787.

Praneetvatakul, S., Schreinemachers, P., Pananurak, P., & Tipraqsa, P. (2013). Pesticides, External costs and policy options for Thai agriculture. *Environmental Science and Policy*, *27*, 103-113.

Prasartkaew, W., Thanomsit, C., & Nanthanawat, P. (2016). Cross-reactivity testing of monoclonal antibody against vitellogenin from Asian seabass. In *Proceedings The 8th Science Research Conference 30-31 May 2016* (pp. 492-497). Phayao: University of Phayao.

Rakhi, S. F., Mohsinul Reza, A. H. M., Hossen, M. S., & Hossain, Z. (2013). Alterations in histopathological features and brain acetylcholinesterase activity in stinging catfish *Heteropneus fossilis* exposed to polluted river water. *International aquatic research, 5*(7), 1-18.

Thanomsit, C., Wattanakornsiri, A. & Nanthanawat, P. (2016). Effects of Glyphosate on Fish Behavior and Histological Alteration of Gills in Asian Sea Bass (*Lates calcarifer*). *Burapha Science Journal*, *21*(2), 204-215.

You, H., Goben, G., N., Du, X., Pali, G., Cai, P., Jones, M., & McManus, D, P. (2016). Functional characterization of *Schistosoma japonicum* acetylcholinesterase. *Parasites & Vector*, *9*(328), 1-12.

Thanomsit, C., Maprajuab, A., Prasartkaew, W., Ocharoen, Y., Wattakornsiri, A., Nanuam, J., & Nanthanawat, P. (2017). Application of Acetylcholinesterase as biomarker for pesticide exposure to reduce health risk in consuming Pond snail and Golden apple snail. Proceeding of 8th Innovation and Technology conferences (pp. A221-A227). Surin: Rajamangala University of Technology Isan Surin Campus.

Thanomsit, C., Maprajub, A., Saowakoon, S., Prasatkaew, W., Ocharore, Y., Wattanakornsiri, A., Nanthanawat, P. (2018). 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. *Thai Journal of Agricultural Science*, *51*(3), 104-117.

Walker, C. H., Hopkin, S. P., & Peakall, D. B. (2006). *Principle of Ecotoxicology.* USA: Taylor & Francis.