

# Potential use of cholinesterase activity from tropical green mussel, *Perna viridis* as a biomarker in effect-based marine monitoring in Indonesia

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**Abstract**—Green mussels (*Perna viridis*) play an important economic and ecological role in the coastal areas of Indonesia. *P. viridis* has been used as an eco-sentinel organism for marine biomonitoring program in Asia regions. To magnify its competency as a relevant organism in biomonitoring, the cholinesterases of *P. viridis* from a selected coastal area of Indonesia have been characterized. Dissected organs which were gill, foot, mantle and posterior adductor muscle were examined for substrate specificity and inhibitors sensitivity using selective and non-selective substrates and inhibitors. The highest level of cholinesterase activity was observed in gill and followed by foot, mantle and posterior adductor muscle. The substrate specificity approach using various alkylthiocholines indicated that the cholinesterases of *P. viridis* constitute an acetylcholinesterase (AChE) and a butyrylcholinesterase (BuChE). Likewise, the inhibitors sensitivity approach using eserine, BW284C51, and iso-OMPA sustained the previous approach which recorded typical AChE and atypical BuChE. The application of cholinesterase activity in three selected areas of Indonesian waters suggested that the gill of *P. viridis* is the most suitable organ for employing the ChE activity as a biomarker in effect-based marine monitoring.

**Key words:** Acetylcholinesterase, biomarker, butyrylcholinesterase, green mussel, monitoring

## Introduction

Cholinesterases (ChEs) are enzymes, which play a major role for degrading one of important neurotransmitters, acetylcholine (ACh) in the synaptic cleft. Degradation of ACh is vital not only to prevent undesired activation of neighbouring neuron or muscle cells, but also to ensure proper timing of signaling to the post-synaptic cell (Chang and Strichartz 2005). The occurrence of two types of ChEs, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) and their variants, have been characterized and recorded in the marine mussel body (Bocquene et al. 1990, Talesa et al. 2001, Brown et al. 2004).

There are two approaches that can be used to characterize ChEs types which are based on substrates and inhibitors selectivity (Sturm et al. 1999). Based on substrates, AChE preferentially hydrolyzes acetylcholine rather than other choline esters and shows substrate inhibition at high concentration. On the other hand, BuChE preferentially hydrolyzes butyrylcholine, but it also hydrolyzes acetylcholine and other choline esters (Massoulie et al. 1993, Chuiko 2000), and does not show substrate inhibition at high concentration (Legay 2000). Furthermore, the ChEs types can also be separated by using their specific inhibitors. Eserine is considered

as a non-selective inhibitor for ChEs, while 1,5-bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284C51) and tetraisopropyl pyrophosphoramidate (iso-OMPA) are considered as selective inhibitors for AChE and BuChE, respectively (Brown et al. 2004).

Enormous attentions have been paid on the ChE activity of mussels from the temperate regions as a biomarker in many biomonitoring programs of marine environment (Mora et al. 1999, Nicholson and Lam 2005). ChEs of some tropical shellfishes which were proposed as eco-sentinel organisms have been classified (Monserrat et al. 2002) and used as a biomarker in marine monitoring (Owen et al. 2002, Lau and Wong 2003). Nevertheless, in my knowledge characterization of ChEs from *P. viridis* that play a vital economic and ecological role in the tropical region has not been established so far. Regarding the ChE activity, a direct application of techniques derived from temperate animals such as blue mussel (*M. edulis*) to tropical green mussel (*P. viridis*) in terms of biomarker in marine pollution monitoring shall be performed carefully. It may bring up genetic and geographic complications. For instance, Valbonesi et al. (2003) reported significantly different levels of the ChE activity of two bivalves, *Ostrea edulis* and *M. galloprovincialis*, while no detectable ChE activity was observed in *Tapes philippinarum*. The sen-

sitivity of the ChE activity from different species of bivalves to ChE-inhibitors is also different (Monserrat et al. 2002). Likewise, some aquatic organisms occasionally have atypical ChEs (Rodryguez-Fuentes and Gold-Bouchot 2004). The presence of atypical ChEs may mask the existence of pesticides effect on the ChE activity of sentinel organisms. In this context, the characterization of ChEs has to be established prior to a monitoring program to avoid erroneous interpretations of data (Bocquene et al. 1990, Sturm et al. 1999, Rodryguez-Fuentes and Gold-Bouchot 2004). Therefore, a research on green mussel (*P. viridis*) ChEs characterization is necessary and intriguing for underpinning the utilizing of the ChE activity in effect-based monitoring.

In some coastal areas of Indonesia such as Jakarta Bay, green mussels (*P. viridis*) play a significant role in small-scale farmer livelihood as marine culture organisms. Nowadays, these small-scale marine cultures are threatened by industrial and agricultural activities, which discharged xenobiotic waste such as pesticides into marine ecosystem (Munawir 2005). Meanwhile, the use of comfortable tools like biomarkers in marine biomonitoring for managing and protecting marine ecosystem has not been conducted yet. It is due to scarcity of information regarding a basic knowledge on biomarkers when considering tropical animals as an ec-sentinel organism. Therefore, the aim of this study is to characterize the ChEs of *P. viridis* from Indonesian waters as a rational basis of the ChE activity employment as a biomarker of organophosphorus and carbamate pesticides and other ChE-inhibitors effects.

## Materials and Methods

### Chemicals

Acetylthiocholine iodide (ASCh), acetyl- $\beta$ -methylthiocholine iodide (A- $\beta$ -MSCh), butyrylthiocholine iodide (BuSCh), physogtigrimine (eserine), BW284C51 (1,5-bis (4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide), iso-OMPA (tetraisopropyl pyrophosphoramidate), 5,5'-Dithio-bis-(-2-Nitrobenzoic acid) (DTNB) were purchased from Sigma. All others reagents used were analytical grade products.

### Sample Collection and Preparation

The study was conducted on expected clean coastal area of Pangkajene Kepulauan regency (ST 1) in South Sulawesi (Fig. 1) and in two sites of Jakarta Bay, namely Kamal Muara (ST 2) and Cilincing (ST 3) (Fig. 2). ST 1 was chosen as reference site because there are relatively few anthropogenic activities performed in this place such as traditional fisheries using static fishing equipment. Those equipments not only attract the target fish, but also attract undomesticated green mussels (*P. viridis*) which attach and dwell naturally on them.

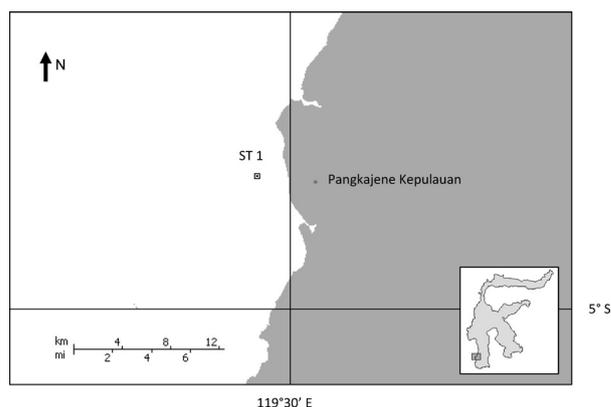


Fig. 1. Sampling station (ST 1) in Pangkajene Kepulauan.

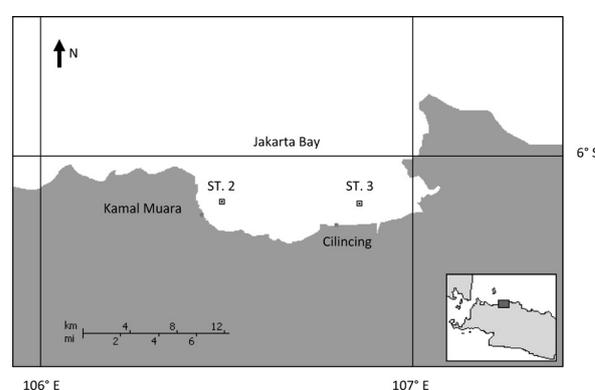


Fig. 2. Sampling stations in Jakarta bay, Kamal Muara (ST 2) and Cilincing (ST 3).

In contrast, the Jakarta Bay is well-known place that heavily polluted by anthropogenic waste from inside and surrounding cities of Jakarta which discharged through 13 rivers. The green mussels were cultivated traditionally in several palces of Jakarta Bay particularly in Kamal Muara and Cilincing. Therefore, the places are of interest as hot spot sites for conducting effect-based monitoring.

Sixteen green mussels (5–6 cm) were handpicked from ST1 for characterization study. The mussels were directly transferred to the laboratory of Marine Science and Fisheries Faculty, Hasanuddin University, Makassar Indonesia using cool box under humid condition. Gill, foot, mantle and posterior adductor muscle (PAM) were cut off, blotted dry and weighted before being placed in 2 ml eppendorf tube containing potassium phosphate buffer (0.1 M/pH 8.0). The tissues were stored at  $-70^{\circ}\text{C}$  before transferred to the Ecotoxicology Department Laboratory, Technische Universitaet of Berlin, Germany using cool box filled with dry ice. In addition, 32 green mussels were collected from the three localities of Indonesia, for effect-based monitoring study. For this purpose only gill of the animals were dissected out and treated as explained above.

A Dounce homogenizer was used to homogenize 0.3 g of each tissue in 2 ml potassium phosphate buffer (0.1 M/pH

**Table 1.** The various alkylthiocholine substrates and cholinesterase inhibitors

Enzyme	Substrate		Inhibitor	
	Selective	Non-selective	Selective	Non-selective
Acetylcholinesterase AChE (EC 3.1.1.7)	Acetyl- $\beta$ -methylthiocholine (A- $\beta$ -MSCh)	Acetylthiocholine(ASCh)	BW284C51	Eserine
Butyrylcholinesterase BChE (EC 3.1.1.8)	Butyrylthiocholine (BuSCh)		Iso-OMPA	

8.0). The homogenate was centrifuged for 10 min at  $10000\times g$  and the supernatant was harvested and stored at  $-80^{\circ}\text{C}$  prior to analysis of the ChE activity and the protein content. The supernatant was diluted in 1:2 of potassium phosphate buffer (0.1 M/pH 8.0) following the enzyme measurement.

### Cholinesterase Activity

The enzyme activity was measured on a 96-well plate following the Ellman method (Ellman et al. 1961) and adapted for microplate. The enzyme measurement was carried out by various alkylthiol substrates in potassium phosphate buffer (0.1 M/pH 8.0) at  $25^{\circ}\text{C}$  containing 0.75 mM 5,5'-Dithio-bis-(2-Nitrobenzoic acid) as a reagent. Subsequently, the enzymatic reaction rate was determined by photometry for microtiter plate (Spectra Thermo TECAN) in an interval of 30 s for 5 min at 405 nm. In each experiment, two blank sections were included to quantify the reaction of thiols with DTNB and of spontaneous substrate hydrolysis sequentially. Three alkylthiocholine substrates, ASCh, A- $\beta$ -MSCh and BuSCh which are selective for ChEs, AChE and BuChE were used to determine cholinesterase types. Substrate kinetic was observed in gill supernatant by utilizing substrate concentrations (ASCh) in the range of 0–12 mM. Inhibitor differentiation approach was also used by employing eserine, BW284C51 and iso-OMPA which are selective for ChE, AChE and BuChE respectively. Incubation of enzymes preparation with 10  $\mu\text{M}$  inhibitors for 30 min was performed prior to determination of residual activities as described before with 3 mM alkylthiocholine substrates. The various alkylthiocholine substrates and cholinesterase inhibitors that were used in this study according to Brown et al. (2004) were presented in Table 1.

### Protein Measurement

A protein content measurement was carried out by diluting gill extract 1:10 with distilled water. It was measured previously by placing 10  $\mu\text{l}$  of the diluted extract and 10  $\mu\text{l}$  of serial dilutions of  $\gamma$ -globuline protein standard into separate well section of the microplate. A blank standard was made by placing 10  $\mu\text{l}$  of distilled water into a blank section of the microplate. After the addition of 5% Bradford-reagent solution (200  $\mu\text{l}$ ) into the microplate, the samples were left in room temperature for 20 min to allow color development. The ab-

sorbance was read at 620 nm using photometry (Spectra Thermo TECAN).

### Data Analysis

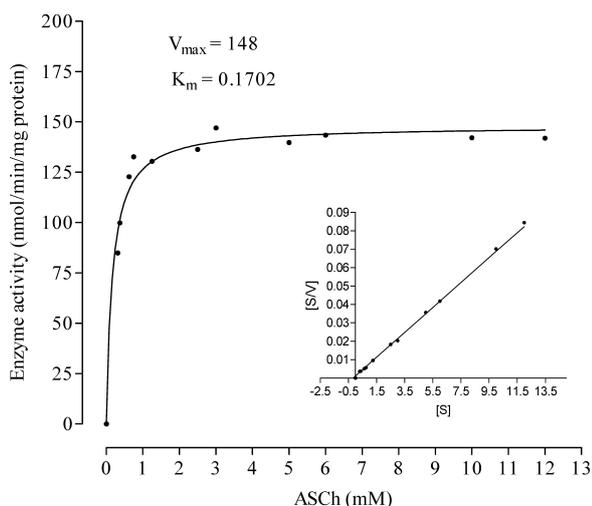
Determination of enzyme kinetic parameters of the ChE activity was performed in green mussel gill. The GraphPad Prism version 4.00 was used to determine enzyme kinetic parameters of ChEs that were  $K_m$  and  $V_{\max}$  by plotting substrate concentrations to the enzyme activity in the term of a nonlinear regression analysis, where a hyperbola was fitted directly to substrate-velocity data. A Hanes-Woolf plot was created by plotting substrate concentrations data [S] toward [S/V] data. The line based on nonlinear regression analysis was overlaid in the plot to provide the best picture of estimating the enzyme kinetic parameters.

The statistical analyses were conducted using the GraphPad Prism version 4.00 edition software to determine the mean difference of the experiments (ANOVA). The Tukey test was used to distinguish the differences between experiments. All determination were conducted in four replicates and expressed as mean and standard deviation. Non-parametric test, Kruskal-Wallis was used to distinguish the difference of ChE activity of the green mussels among the sites. If there were differences among the sites, the test was continued by Dunn's multiple comparison test to determine the difference between two sites.

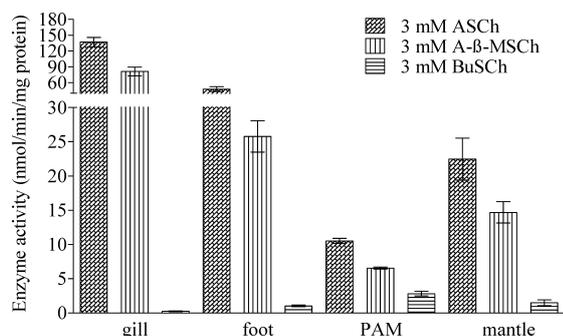
## Results

### Enzyme Kinetic

The kinetic constants that are  $K_m$  and  $V_{\max}$  were determined by plotting the enzyme activity of gill mussel supernatant against substrate concentrations (ASCh) (Fig. 3). By employing substrate concentrations in the range of 0–12 mM, the  $K_m$  and  $V_{\max}$  value were 0.1702 mM and 148.0 nmol/min/mg protein respectively. In light of tested concentrations, the ChE activity obeyed Michaelis-Menten behavior showing an indication of substrate inhibition. The transformation data from the Michaelis-Menten plot using the Hanes-Woolf method showed a linearity of the line ( $R=0.999$ ). The results also showed that the maximum activity of the enzyme was achieved when the substrate concen-



**Fig. 3.** Effect of acetylthiocholine (ASCh) concentration as substrate on esterase activity in supernatant extract 10000 g ( $S_{10}$ ) from gill *P. viridis*. Insert is Hanes-Woolf-plot.



**Fig. 4.** Comparison of esterase activity of various tissue of green mussel with various substrates (3mM). Pooled organs from sixteen green mussels were measured for the enzyme activity (nmol/min/mg of protein). PAM=posterior adductor muscle.

tration was 3 mM (Fig. 3). Accordingly, this concentration was used for further determination.

**Substrate-Based characterization**

The substrate-based characterization of ChEs enzymes have been studied by using three substrate analogues, which are ASCh, A-β-MSCh, and BuSCh. The results depicted that the highest ChE activity was recorded in the gill followed by foot, mantle, and PAM when using ASCh and A-β-MSCh as substrates (Fig. 4). In contrast, with BuSCh as a substrate, the gill had the lowest activity, while PAM presented the highest activity significantly succeeded by mantle and foot. Nevertheless, the highest ChE activity was generally detected when the enzymes were incubated with ASCh, while BuSCh substrate showed the lowest reaction with the enzymes. Compared to BuSCh as substrate, A-β-MSCh revealed a significant stronger reaction with the enzymes (Fig. 4). In all organs, incubation of the enzymes by A-β-MSCh substrate exhibited enzymes activities relative to ASCh substrate ranging from 30.67–65.40% (Table 2). Accordingly, with BuSCh as a substrate the enzyme activity relative to ASCh substrate showed the percentage of activity ranging from 0.18–26.79% (Table 2). The results indicated that the true AChE constituted dominantly in four examined organs i.e., gill, foot, mantle, and PAM, while the BuChE coexisted in those four organs. Eventually, the existence of BuChE activity in PAM was observed higher compare to other organs.

**Inhibitor-Based Characterization**

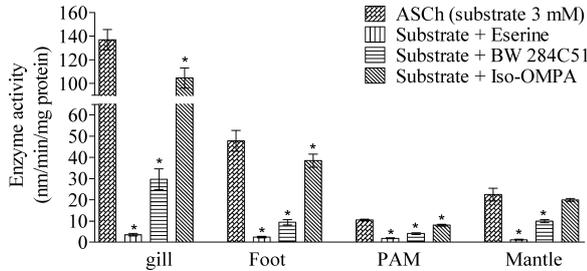
Three inhibitors which assumed to be an unselective (eserine) and selective (BW 284C51 and iso-OMPA) inhibitors of ChEs were used to confirm the substrate-based characterization of ChEs (Fig. 5, Fig. 6, & Fig. 7). The figures demon-

**Table 2.** Mean of cholinesterase activity of three substrates as measured in various organs of green mussel with comparison to ASCh

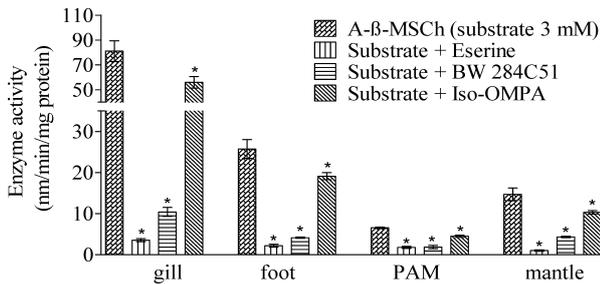
Substrates	ChEs activity (nmol/min/mg P)	Enzyme activity relative to ASCh (%)	Significantly less than ASCh at $p < 0.01$
<b>Gill</b>			
Acetylthiocholine (ASCh)	137.03±8.6	100	
Acetyl-β-methylthiocholine (A-β-MSCh)	81.10±8.30	59.18	Yes
Butyrylthiocholine (BuSCh)	0.25±0.06	0.18	Yes
<b>Foot</b>			
Acetylthiocholine (ASCh)	47.82±5.44	100	
Acetyl-β-methylthiocholine (A-β-MSCh)	25.75±2.29	53.86	Yes
Butyrylthiocholine (BuSCh)	1.02±1.14	2.13	Yes
<b>Posterior adductor muscle</b>			
Acetylthiocholine (ASCh)	10.50±0.31	100	
Acetyl-β-methylthiocholine (A-β-MSCh)	6.55±0.16	62.34	Yes
Butyrylthiocholine (BuSCh)	2.81±0.36	26.79	Yes
<b>Mantle</b>			
Acetylthiocholine (ASCh)	22.46±3.07	100	
Acetyl-β-methylthiocholine (A-β-MSCh)	14.69±1.55	65.4	Yes
Butyrylthiocholine (BuSCh)	1.48±0.44	6.61	Yes

strated the usefulness of the used inhibitors in characterizing ChE enzymes from different organs of *P. viridis*.

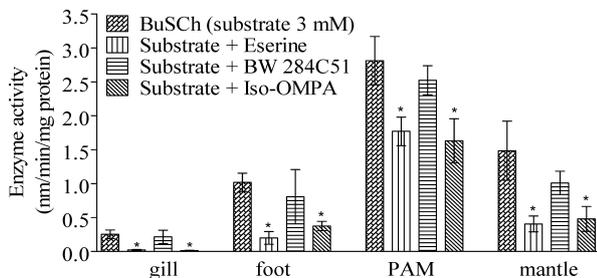
Fig. 5 showed that the enzyme using ASCh as substrate was inhibited by three different inhibitors. Eserine as general inhibitor for ChEs was able to inhibit the enzyme activity almost completely. A strong inhibition of the enzyme activity



**Fig. 5.** ChE activity of various organs of green mussel with acetylthiocholine (ASCh) as substrate and the inhibitors (10  $\mu$ M); Eserine, BW 284C51 and Iso-OMPA. The enzyme activity was measured by pooling organs from sixteen green mussels (nmol/min/mg of protein). Bars indicated standard deviation. \* Significantly different from control ( $p < 0.01$ ). PAM=posterior adductor muscle.



**Fig. 6.** ChE activity of various organs of green mussel with acetyl beta methyl thiocholine (A- $\beta$ -MSCh) as substrate and the inhibitors (10  $\mu$ M); Eserine, BW 284C51 and Iso-OMPA. The enzyme activity was measured by pooling organs from sixteen green mussels (nmol/min/mg of protein). Bars indicated standard deviation (SD). \*Significantly different from control ( $p < 0.01$ ). PAM=posterior adductor muscle.



**Fig. 7.** ChE activity of various organs of green mussel with Butyrylthiocholine (BuSCh) as substrate and the inhibitors (10  $\mu$ M); Eserine, BW284C51 and Iso-OMPA. The enzyme activity was measured by pooling organs from sixteen green mussels (nmol/min/mg of protein). Bars indicated standard deviation (SD). \* Significantly different from control ( $p < 0.01$ ).

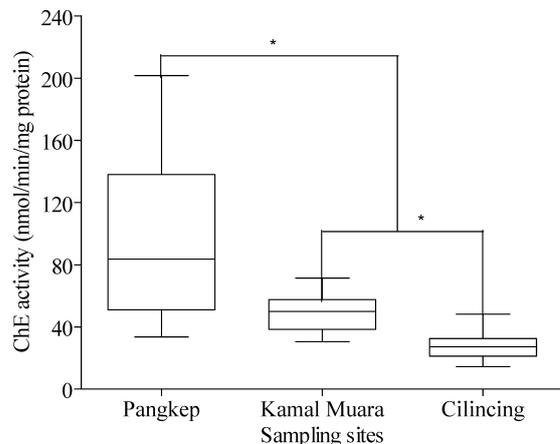
was still demonstrated by specific inhibitor for AChE i.e., BW284C51. On the other hand, iso-OMPA as specific inhibitor for BuChE delineated a weak inhibition, although it was statistically significant.

Interesting results were obtained when A- $\beta$ -MSCh was used as substrate for ChEs (Fig. 6). As general inhibitor eserine inhibited the enzyme almost entirely. Moreover, as specific inhibitor for AChE, BW284C51 inhibited the enzyme activity strongly. A partial inhibition of the enzyme activity was demonstrated by specific inhibitor of BuChE, iso-OMPA. It was intriguing since the ability of iso-OMPA inhibiting the enzyme activity led to a question on its specificity or a variant of BuChE.

Iso-Ompa as specific inhibitor of BuChE delineated a strong inhibition as strong as the inhibition by eserine when BuSCh was used as substrate (Fig. 7). In contrast, specific inhibitor for AChE, BW284C51 did not inhibit the enzyme. It showed the specificity of BW284C51 as specific inhibitor of AChE.

**Effect-based Monitoring**

Statistical analysis showed the difference of ChE activity in the gills of the samples ( $p < 0.05$ ) (Fig. 8). The animals collected from the reference site had the significant highest ChE activity (83.56 nmol/min/mg protein) followed by the green mussel collected from heavily polluted areas, Kamal Muara (49.92 nmol/min/mg protein) and Cilincing (27.20 nmol/min/mg protein). Between two heavily polluted sites, the animals inhabited in Kamal Muara showed significant less inhibition of the ChE activity than those from Cilincing ( $p < 0.05$ ).



**Fig. 8.** ChE activity of green mussel gill, *Perna viridis* from the pollution gradient of Indonesian waters. Kamal Muara and Cilincing are expected as polluted areas, while Pangkep is expected as a clean area. Data were expressed as median (25% and 75% quartile, 5% and 95% confidence interval). \* indicate the different enzyme activity among the sampling sites ( $p < 0.05$ ).

## Discussion

This current study attempted to characterize ChE types based on substrate hydrolysis and sensitivity to inhibitors in different organs of tropical green mussel (*P. viridis*) from expected clean coastal area of Indonesia. A measurable ChE activity from *P. viridis* organs resulted from the present study is considered to be as a rational basis of effect-based marine biomonitoring programs of neurotoxic substances.

### Substrate-Based Characterization

Characterization of ChE types of *P. viridis* using various alkylthiol substrates was preceded by measuring enzymes kinetic parameters. The hyperbolic regression analysis of the enzymes kinetic using ASCh as a substrate demonstrated that the ChE activity of gill conformed Michaelis-Menten plot behavior (Fig. 3). An apparent  $K_m$  value was 0.148 mM, which was in the same range of those reported for ChEs in some aquatic animals (Table 3). Compared to the  $K_m$  value of other bivalves, which have been reported in the previous studies, ChEs of *P. viridis* revealed the higher  $K_m$  value. The results showed that ChEs of *P. viridis* gill reached a maximum velocity ( $V_{max}$ ) at level higher than that of other reported bivalves. The  $V_{max}$  was observed at 3 mM substrate concentration and used for further experiments.

By the fact that  $K_m$  and  $V_{max}$  are properties of individual enzyme, these factors are not very useful parameters for comparing enzymes. A  $K_m$  reflects both binding of enzyme-substrate and the catalytic constant of the enzyme catalyzed reaction, while a  $V_{max}$  depends on a catalytic constant. The more informative parameter for comparing enzyme is the ratio between  $V_{max}$  and  $K_m$  ( $V_{max}/K_m$ ) which reflects the efficiency of the enzyme for catalyzing its reaction (catalytic efficiency). The present work revealed that  $V_{max}/K_m$  value of *P. viridis* gill esterase was higher than those observed in previous studies (Table 3). The results of the present study suggested that the ChEs of *P. viridis* gill catalyzed the substrate more effective than those of other bivalves catalyzed and might reflect the higher ChEs activity compared to other bi-

valves. By comparing two bivalves Valbonesi et al. (2003) observed that the bivalve with a higher  $V_{max}/K_m$  value revealed a higher ChE activity. Nevertheless, the comparison of ChE activity data should be carried out carefully since it was often masked by differences between either sample preparation methods or the use of varying arbitrary units to describe the data (Brown et al. 2004). Therefore, inter-calibration among laboratories, which perform ChEs assay is needed.

Concerning biomonitoring of neurotoxicants in a laboratory and field scales, utilizing of enzyme kinetic parameters viz.  $K_m$ ,  $V_{max}$  and  $V_{max}/K_m$  on data interpretation of ChE activity as a biomarker would be a useful. In term of insect, Zhu and Gao (1999) found strong evidence that resistant strain of green bug (*Schizaphis graminum*) possessed high ChE activity associated with low affinity and high catalytic activity, and low inhibition to paraoxon as compared to ChE activity from the susceptible strain. The increasing activity of ChE induced by pesticides was also observed in the laboratory scale from larvae of the grass shrimp, *Palaemonetes pugio* (Key and Fulton 1993) and freshwater mussel, *Elliptio complanata* (Moulton et al. 1996). Romani et al. (2005) reported that organophosphorus pesticide, chlorpyrifos, caused an elevation of ChEs activity of the bivalve, *Scapharca inaequivalvis* at sublethal concentration instead of producing inhibition of the enzymes activity. The evidence will be confounding factors in application of ChEs activity as a biomarker if the  $K_m$ ,  $V_{max}$  and  $V_{max}/K_m$  are not taken into account since theoretically ChEs activity can be inhibited by organophosphorous pesticides. In addition, Romani et al. (2005) observed that the increase of ChE activity after the chlorpyrifos exposure was linked to a significant increase in the synthesis of AChE-specific mRNAs followed by decreasing enzyme catalytic efficiency ( $V_{max}/K_m$ ). This evidence indicated a resistance of the bivalve to the pesticide after being exposed to chlorpyrifos (Romani et al. 2005), as the mechanism of resistance is mediated by amplification of esterase genes and often accompanied by a modification of kinetic parameters of acetylcholine hydrolysis (Fournier et al. 1992, Zhu and Gao 1999).

**Table 3.** ChE kinetic parameters from aquatic animals

Animals	Sources	$V_{max}$ (nmol/min/ mg protein)	$K_m$ (mM)	$V_{max}/K_m$ ( $\text{min}^{-1}$ )	References
<i>Aqtinia equina</i>	gill	216	0.098	$2.20408 \times 10^{-3}$	Talesa et. al. (1996)
<i>Spongia officinalis</i>	gill	142	8	$1.775 \times 10^{-5}$	Talesa et. al. (1996)
<i>Crassostrearhizophorae</i>	gill	1.92	0.046	$4.1739 \times 10^{-5}$	Alves et al. (2002)
<i>O. edulis</i>	gill	4.82	0.093	$5.1828 \times 10^{-5}$	Valbonesi et al. (2003)
<i>M. Galloprovicianlis</i>	gill	18.36	0.076	$2.4158 \times 10^{-4}$	Valbonesi et al. (2003)
<i>M. edulis</i>	gill	9.198	0.013	$7.0754 \times 10^{-4}$	Mora et al. (1999a)
<i>P. viridis</i>	gill	148	0.1702	$8.6957 \times 10^{-4}$	Present study
<i>Chasmagnathus granulata</i>	Thoracic ganglia	1750	0.28	$6.25 \times 10^{-3}$	Monserat et al. (1998)

The substrate-based study revealed that tested organs of *P. viridis* consisted of two ChEs types, AChE and BuChE with AChE dominating because A- $\beta$ -MSCh substrate revealed a higher activity of the enzyme than BuSCh substrate. By considering hydrolysis of ASCh in the tissue extract as a total activity of ChEs enzymes, the activity of AChE only achieved 59% of the total activity (Table 2). The rest is BuChE and may be propionylcholinesterase (PrChE). This result was in agreement with a previous study that reported an atypical of ChEs enzyme from 'soluble' fraction of *M. edulis* gill (Brown et al. 2004). The presence of AChE and BuChE in gill of brown mussel *P. perna* was also reported by Alves et al. (2002).

The distribution of two ChEs enzymes were observed variously in tested organs. The results showed that AChE activity was detected dominantly in all tested organs, which were investigated in the following order: gill>foot>mantle>PAM (Table 2, Fig. 4). In contrast, the highest activity of BuChE was observed in PAM followed by mantle, foot and gill. The highest activity of AChE in gill observed in this present work was in agreement with previous studies, which were conducted in gill of European counterpart of green mussel i.e. *M. edulis* (Bocquene et al. 1990) and *M. galloprovincialis* (Escartin and Porte 1997). The dominant of the AChE activity from gill of *M. galloprovincialis* was about 6-fold compared to the AChE activity from digestive gland has been also reported (Porte et al. 2001). However, Najimi et al. (1997) reported a high AChE activity observed in the digestive gland and muscle of African brown mussel, *P. perna*.

### Inhibitor-Based Characterization

The inhibitor-based characterization has been conducted to confirm and complete the ChEs classification based-on substrates (Sturm et al. 1999). The presences of AChE and BuChE in all tissues of *P. viridis* have been demonstrated in this work and seemed to be strengthened by the inhibitor approach. The strong inhibition of eserine on all substrates hydrolysis in all tissues indicated that all tested organs of *P. viridis* contained ChEs, which confirmed the non-selectivity of eserine. Further classifying experiments by using two specific inhibitors, BW284C51 and iso-OMPA were considered as sensitive to AChE and BuChE respectively sustained what observed on the substrate-based study with little deviations. The extract tissues, which hydrolyzed A- $\beta$ -MSCh substrate was not only sensitive to BW284C51, but also sensitive to iso-OMPA. Furthermore, the inhibition of iso-OMPA on A- $\beta$ -MSCh hydrolysis is significantly lower than that of BW284C5, which indicated a partial inhibition of iso-OMPA on that specific substrate. On the other hand, BuChE of all tissues was sensitive to iso-OMPA and did not demonstrate sensitivity to BW284C51. The results was questioning the specificity of iso-OMPA, which considered as specific inhibitor of BuChE or pointed out the existence of an atypical

BuChE in studied organs of *P. viridis*. Nevertheless, the question of iso-OMPA specificity was weakened by the fact that inhibition of iso-OMPA was partial. If iso-OMPA was non-specific inhibitor, it inhibited all substrates hydrolysis almost completely similar as eserine. Hence, the most acceptable explanation is the presence of an atypical BuChE in the studied organs of *P. viridis*. The capability of an atypical BuChE hydrolyzing both BuSCh and A- $\beta$ -MSCh substrates, which were inhibited by iso-OMPA has been reported as well by Sturm et al. (1999) in marine teleost fish. The hypothesis was also supported by the fact that the atypical BuChE of *P. viridis* tissue was not sensitive to BW284C51, the specific inhibitor of AChE. Consequently, the insensitivity of the enzyme to anti-AChE could be postulated as a characteristic of an atypical BuChE which has a lower affinity for many inhibitors and unnatural substrates since as an anti-AChE scavenger BuChE has broad specificity so that every anti-AChE is also anti-BuChE (Soreq and Glick 2000). Eventually, it is stated clearly that the inhibitor-based characterization was highlighted by substrate-based strategy for classifying ChEs, which identified at least two types of ChEs namely typical AChE and atypical BuChE were observed in studied organ of *P. viridis*.

Regarding marine biomonitoring using ASCh substrate, which is the unselective substrate of ChEs activity of *P. viridis* is preferable since it describes both true (AChE) and pseudo cholinesterase (BuChE). Furthermore, the occurrence of the atypical BuChE in *P. viridis* tissue may amplify the use of ChEs activity from this animal as a valuable tool for pesticides effect-based monitoring since atypical BuChE has a lower affinity for many inhibitors (Soreq and Glick 2000). Subsequently, the possibility of the BuChE confounds the data interpretation by scavenging the contaminants before reaching ChEs will be reduced. The omnipotence of discharged pesticides effects on studied organisms are not diminished by the scavenger activities so that the type II error can be avoided. Likewise, the present study revealed that gill has the highest ChE activity, which emphasized the employment of this organ as a suitable organ for risk-based monitoring tool. The advantage of this organ in term of biomarker study has also been strengthened by Lau et al. (2004), who found less sensitivity of protein of *P. viridis* gill to seasonal variation that produced higher protein compared to the whole body tissues.

Porte and Albaiges (2002) demonstrated that the ChE activity from the gill of blue mussels (*M. galloprovincialis*) was more sensitive than that of digestive gland and it revealed a certain correlation with the concentration of fenitrothion in whole mussels. It has been reported that the gill of *P. viridis* which were collected from Hong Kong waters had higher ChE activity than that of the whole tissue and this ChE activity was not size-dependent (Lau and Wong 2003). This is conceivable because mussels use their gills not only

as a respiratory apparatus but also as filter feeder organ thereby ambient water filtered and managed for gaseous exchanges and sifting food (Bayne et al. 1976). Since the gill are in the front line of contact with contaminants and the first line of defense (Lau and Wong 2003), detoxification compounds such as ChEs are necessary to be produced to protect other organs. Consequently, the production of ChEs not only control neurotransmission, but also serves as contaminants detoxification particularly for organophosphorus and carbamate pesticides (Soreq and Seidman 2001). Those evidence set up the gill as a *par excellence* tissue for biomarkers application to minimize effects caused by the natural reproductive cycles and the dilution effect due to large variation in the total tissue protein (Lau et al. 2004). The selection of the gill as tissue target for conducting biomarker studies were also shown by the nature of the gill, which comes into contacts with relatively large volumes of seawater compared to the rest of the animal so that conferring them with the potential for being a suitable target tissue for xenobiotic substance exposure.

In consequence, the present study employed the gill of *P. viridis* to investigate pollutants effect to ChE activity in gradient pollution of Indonesian coastal area. The results indicated that the ChE activity of the gill of *P. viridis* from pristine site was significantly higher than that from the gill of *P. viridis* living in polluted sites (Fig. 8). The reduction of the ChE activity from the gill of *P. viridis* collected from Kamal Muara was about 49.2%. Statistically, the greatest reduction of the ChE activity was indicated in mussels from Cilincing, which was about 72.41%. By exposing brown mussels (*Perna perna*) to furadan (carbamate pesticide), Alves et al. (2002) observed the ChE activity of the gill was suppressed by 35%. Therefore, it is suggested that the ChE activity is a sensitive tool since it can in fact distinguish gradient levels of two heavily polluted areas.

## Conclusion

The present study revealed that ChEs from *P. viridis* organs consist of a typical AChE and an atypical BuChE. The typical AChE dominates in the gill, while the atypical BuChE occurs dominantly in PAM. In addition, the highest ChE activity of the gill emphasized the employment of this organ as a suitable organ for effect-based monitoring and the ChE activity provides a useful biomarker for effect-based monitoring in coastal areas of Indonesia. As a biomarker, the ChE activity from the gill of *P. viridis* has ability to discern different effect of pollutant on cholinergic system of *P. viridis* that populated in the sampling sites.

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