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Biochemical Biomarkers: Fish Cholinesterase Biosensor for Heavy Metals Detection in Aquatic Pollution Monitoring

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Abstract – Recently, the contamination of heavy metals towards the environment especially in aquatic system has drastically increased. Heavy metals are able to transform into persistent metallic compound in which it can be accumulated within the organisms' body system, disrupting the food chain and eventually threatened the human life. The occurrence of heavy metals spillage in the rivers and lakes are due to the careless disposal of excess heavy metals used for human activities. The accumulation of heavy metals in water system will affect all aquatic organisms especially fish. The toxicity of copper in fish can be determined by several changes in the fish under treatment with heavy metals sub-lethal concentration, LC_{50} within 96-hours period of acute exposure. Therefore, fish can be considered as a high potential biomarker for monitoring heavy metals pollution in aquatic system. Several selective organs are highly sensitive to the xenobiotic pollution and express changes to the exposure. One of the most potential biomarker is the biochemical biomarker of cholinesterase (ChE) inhibition by heavy metals in fish has been well studied in pollution monitoring recently. Thus, this paper gives an overview of the manipulation of fish as a biomarker of heavy metals through enzymatic reaction which have proven to be very useful in the environmental pollution monitoring.

Keywords: Biomarker, biosensor, cholinesterase, fish toxicity, heavy metal pollution.

Introduction

Natural environment closely located to the aggressive and excessive urbanisation and industrialisation activities is commonly polluted by contaminants. These pollutants include heavy metals, which cannot be removed by conventional wastewater treatment process and often result in a huge number of recalcitrant in the aquatic environment (de Lima, Rogue, & Almeida, 2013); Yen & Sabeh, 2013). Heavy metals are defined based on their sheen properties as the inorganic metallic elements that are able to bring malicious effects in the form of metal ions with the density of greater than 5 g/cm^3 (Sulaiman, Mustaffa, & Khazaai, 2016; Chen , Tenga, Lu, Wang & Wang, 2015). Since the Iron Age, mankind has acknowledged several applications of heavy metals in making home appliances, water pipes, kitchen applications and weapons. Recently, the rise of advance technology has brought people to the exploration and discovery of many other purposes of heavy metals not only for the physical civilisation, but also for their own benefits (Zaki, Mustafa, & Fawzy, 2015). In the industrialised world that we live in today, conflicts have been raised stating that heavy metals are among the most dangerous pollutants (Ullah & Zorriehzahra, 2014). This is due to the outstanding physical-chemical possession of heavy metals, which has widened their range of usage in industrial processes, fertiliser and biocides production.

Contamination of metals in aquatic system is longing since metals accumulate in aquatic organisms such as fish and transform into persistent metallic compounds (Sercikova et al., 2013; Taweel, Othman & Ahmad, 2011). The accumulated toxic metal components can actually give adverse effects to the aquatic life, which further disrupt the biological food web and eventually threaten the ultimate mankind as the consumer (Yuswir, Praveena, Aris, Ismail & Hashim, 2015; Mashifane & Moyo, 2014). Besides, heavy metals can also give histological effect as well as biochemical changes to aquatic life especially fish. Monitoring the changes occurred in biochemical parameters may offer the early cautionary on general as well as specific toxicological responses. Biochemical biomarkers of contamination are the substantial keys used in fish toxicity tests and field monitoring of aquatic pollution. Meanwhile, the toxicological study on acute exposure has presented that the changes in concentration and activity of some enzymes may reflect cells damage in specific organs (Osman, 2012).

Biochemical biomarker

Biomarker is one of the useful implements in recent environmental assessment as it is able to estimate the pollutants involved during monitoring programme (Kaviraj, Unlu, Gupta & Nemr, 2014). It is also used in various fields including medicine, toxicology, development biology, and basic scientific research. Single organism can be used as biomarker from many different level of organisation from sub-cellular to whole organism for recalcitrant detection. For example, molecular, biochemical, and tissue biomarkers for different chemical detection can be selected from only one organism (Osman, 2013). Fish can be considered as the best biomarker for monitoring the aquatic pollution. Excessive amount of heavy metals can be highly toxic to fish. It is able to accumulate in tissues and poison the fish. Some of the general severe effects include the disruption of vital operation and reproduction of fish, weakened immune system and the induction of pathological changes upon exposure (Authman, Zaki, Khallaf & Abbas, 2015).

The accumulation of heavy metals in tissue leads to the redox reaction catalysis that can generate reactive oxygen species (ROS) (Sabullah et al., 2015b). ROS is an inevitable composite of aerobic system with the ability to channelise its lethality by imparting oxidative stress under a stressful environment and eventually cause the biochemical, molecular, and morphological alterations in fish (David & Kartheek, 2016; El-Gazzar, Ashry & El-Sayed, 2014). Previous studies have proved that physiological and biochemical alterations can be utilised as a highly potential biomarker for monitoring the pollution in aquatic system. Thus, fish was selected as biomarkers since it is closely related to humankind (Kumar, Kumar & Devi, 2015).

The changes of biochemical compound in the fish organs upon exposure have become the significant tools for pollution monitoring in aquatic system as these changes were seen to occur quicker than physiological responses and provide information on the sensitivity of organisms with regard to uptake, biotransformation and detoxification patterns (Ploetz, Fitts & Rice, 2007; Hayat et al., 2014). In addition, these alterations also prove the contact made by specimens with a specific group of xenobiotic components and clarify their future metabolic rate. The activity and concentration of involved enzymatic systems and substrates can be quantified as the extent of their induction or inhibition can also be compared to the control. The response depends on many factors including xenobiotic properties, concentration, the presence of other compounds, length of exposure, water pH and temperature (Haluzová et al., 2011).

There is a previous report mentioning that metabolic enzymes such as aspirate amino transferases (ASAT), alanine amino transferases (ALAT), superoxide dismutase (SOD) and glutathione s-transferase (GST) have significantly increased in the activity induced by toxicants particurlarly heavy metals (Sabullah et al., 2015b). There are also other biochemical alterations that can be potentiallly used as biomakers including the occurrence of metallothionien. In fact, cytosolic detoxificatory protein metallothionien (MT) expression is the most leisurely being discussed as specific biomarker which induced by particular trace metal binding (Khati, Oulia, Mouneyrac & Banaoui, 2012; Naji, Ismail, Kamrani & Sohrabi, 2014).

These non-enzymatic proteins are related with significant cellular protective processes that include the inactivation of hydroxyl radicals and protection against immunotoxins, hematoxins and nephrotoxins. Given these characteristics, MT was suggested as a biomarker for heavy metals pollution. However, the induction of MT is a dependable response to metal contamination as the determination of MT concentration alone can show false positive reaction due to limited availability of MT in the specific organs of various fish species, age and analysed tissues. Moreover, external issues such as season, temperature and diet can also effect the induction of MT (Sevcikova et al., 2013).

Cholinesterases

One of the important elements in environmental analysis to be considered is the direct relationship between ChE inhibition and the behavioural, biochemical, and physiological alterations that occur in exposed organisms. Cholinesterasic inhibition was fund to be closely accompanied with a rise in mortality and survival rates of aquatic organisms impaired along with ChE inhibition when approaching 70% of exposure (Nunes, 2011; Fulton & Key, 2001). Cholinesterase (ChE) is a ubiquitous enzyme from serine hydrolase group that catalyses the hydrolytic cleavage of acyl group from the esters of choline. ChEs are extremely complex; they exist in different forms, expressions, biological function, distribution and catalytic activity (Nunes, 2011).

There are three main types of ChEs that are evolutionary related, but encoded in different genes namely acetylcholinesterase (AChE; E.C. 3.1.1.7), butyrylcholinesterase (BChE; E.C. 3.1.1.8), and propionylcholinesterase (PrChE; E.C. 3.1.1.8). These enzymes have their own specific features including substrate specificity and kinetic parameters that can be determined from their amino acid composition and the conformation of their active centre (Jebali et al., 2013). AChE is also known as specific lytic enzyme for hydrolysing acetylcholine (ACh) to form two inactive products known as acetate and choline. The hydrolysis reaction take place by the nucleophilic attack of the carbonyl C, acylating the enzymes and liberating choline followed by rapid hydrolysis of the acylated enzyme, resulting acetic acid and the renewal of esteratic site. Generally, AChE and BChE present a similar amphiphilic or soluble molecular forms in tissues and body fluids with different tissue distribution and categorised as homo and hetero-oligomeric molecular forms that belonged to α and β protein class. Each monomer is established by 12 β strands and 15 α helices (Çokuğraş, 2003). These homo- and hetero-oligomeric forms are summarised as five different molecular forms such as amphiphilic monomer (Type I), amphiphilic dimer (Type II), hydrophobic-tailed tetramers, collagen-like or asymmetric form and soluble tetrameric form.
Monomer (G_1), dimer (G_2), and tetramer (G_4) forms of ChE are classified into symmetric form or globular form of ChE, which made up the catalytic subunit of ChE. Asymmetric forms of ChE characterise the presence of a collagen-like tail for docking to the basal lamina formed by the triple helical structure of three collagenases that associated with subunits A_4, A_8, and A_{12}.

AChE basically possesses a significantly high specific catalytic activity. Its active site is located at about 4Å from the bottom part of the molecule, which is divided into ‘anionic’ and ‘esteratic’ subsites. Esteratic site comprises the catalytic triads (Ser-His-Glu) amino acids strand, while the anionic site accommodates the positive quaternary pole of acetylcholine (ACh). Anionic site is defined by amino acids Trp, Phe 330, and Phe 331. Additionally, these subsites correspond to catalytic machinery and choline – binding pocket, respectively (Figure 1) (Colovic et al., 2013; Jebali et al., 2013). The difference between AChE and pseudocholinesterases, BChE and PrChE lays in their structure form where the missing of 6 aromatic residues of AChE (Phe 288 and Phe 290) is replaced by amino acids Leu and Val (Çokuğraş, 2003). BChE and PrChE are known as pseudocholinesterase that become less-specific lytic enzymes as they hydrolyse acetylthiocholine iodide (ATC) at minor rate compared to their specific substrates, which are butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) (Hayat et al., 2016; Falugi & Aluigi, 2012).

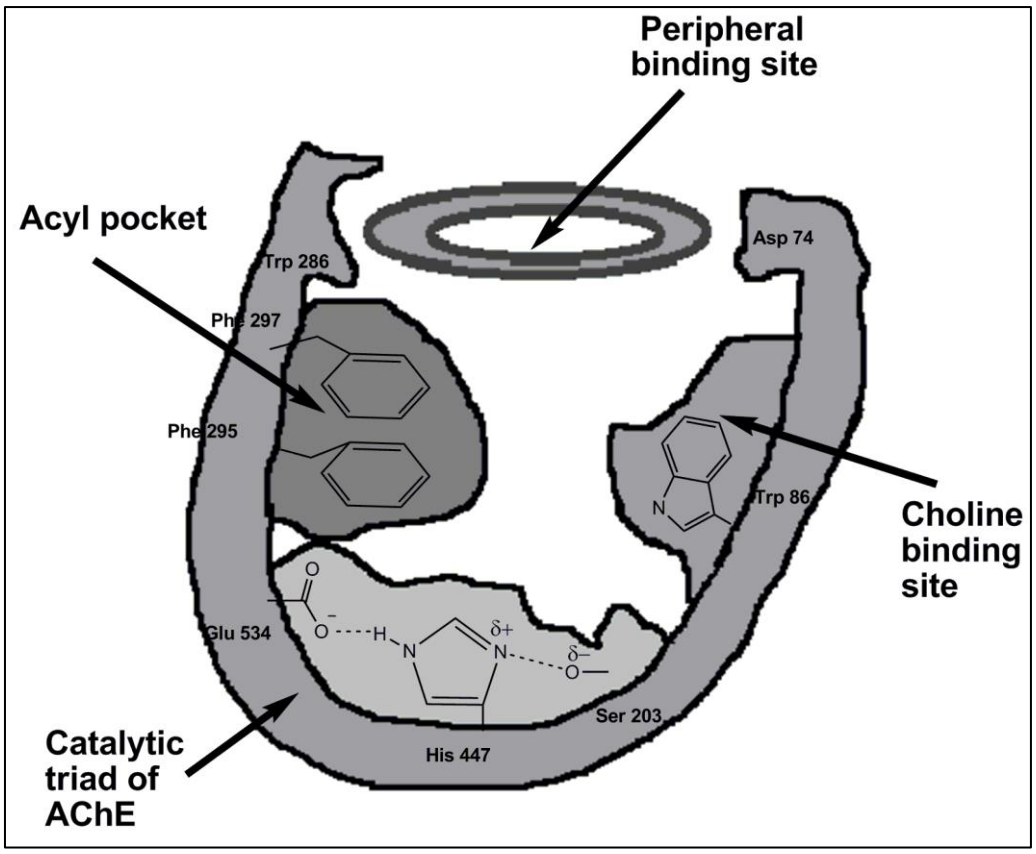

Figure 1: Schematic representation of AChE binding sites (Colovic et al., 2013)

Recently, many studies are focusing to assess ChE inhibition as a pilot screening of pollution monitoring and develop it as a biomarker and biosensor. This is because ChE is reported to a broad range biosensor as it can even screen for several types of inhibitor including pesticides, insecticides, drugs, alphatoxin, detergents and definitely heavy metals such as Cu, Cd, and Hg based on the samples tested on previous studies by Sabullah et al., (2015a), Tham et al., (2008), Frasco (2007), Rodriguéz-Fuentes and Gold-Bouchot (2004), and Glusker (1999). Unlike esterases, AChE inhibition results in an elevation of ACh level that leads to a continuous and excessive stimulation of neural system; this could cause severe effects such as tetany, paralysis and even fatality (Soorya et al., 2012). ChE is selectively inhibited by known inhibitors, organophosphates (OP) and carbamates (CB) (Koenig et al., 2013; Xuereb et al., 2007). Meanwhile, the specific inhibitors for AChE and BChE reported in previous are 1,5-bis-(4-ally dimethyl-aminoniumphenyl-peutan-3-one dibromide (BW 284C51) and tetraisopropyl pyrophosphoramide (iso-OMPA), respectively (Rakhi, Reza, Hossen & Hosain, 2013; Falugi & Aluigi, 2012). However, there has been inadequate specificity found in cholinesterase inhibition by pesticides. There are several compounds discovered with the capability to inhibit them in a way almost blurry at first sight. Even so, Enzyme inhibition patterns demonstrate differently by time for covalent binding and type or duration of recovery. The interaction of anticholinesterasic can be both at active and allosteric sites of the enzyme, expressing mixed-typed inhibition mechanisms (Assis, Castro, Amaral, Carvalho, Carvalho Jr. & Bezerra, 2010). Current researchers have intended to use ChE extracted from fish as a biomarker for heavy metals pollution screening element due to the vast sensitivity of ChE towards a broad range of toxicants such as pesticides, drugs, heavy metals, detergents and alphatoxin (Sabullah et al., 2015b).

ChE was selected as the best enzyme for biomarker and biosensor due to its ability to react or possess the inhibition of exposure through in vitro and in vivo methods (Lionetto, Caricato, Calisi, Giordano & Schettino, 2013). In vitro assay can be conducted by discriminating a component of an organism to provide specific detail analysis. For instance, ChE isolated from *Torpedo californica* and Tambaqui were used to revise the molecular structure including amino acids found in the catalytic triad of enzyme accountable for substrate degradation, while in vivo assay was implemented by a previous

study to evaluate environmental risks such as the effects of metal ion on aquatic organism (Silva et al., 2015; Sabullah et al., 2015b; Pasha et al., 2013). The method would be either exposing the toxicants on fish via acute toxicity testing with different dosage, or the oxidative stress that was induced at the same time by generating highly reactive oxygen species (ROS) leading to cell death programme.

Mechanism of ChE inhibition by metal ion

With regard to the utilisation of cholinesterases as biomarkers, Frasco (2007) obviously demonstrated that the type and effectiveness of inhibition of a cholinesterase by mercury strongly depend on the species. Nevertheless, biomarker tools may not give a detailed result in quantitative data. However, it still serve as a precautionary step prior to second validation through high technology instruments. Therefore, the kinetic characterisation of cholinesterase inhibition in the selected species would be prerequisite for field studies. A biosensor is an alternative to a biomarker where the enzyme is linked to a surface deep in the surveyed solution and records the inhibition of cholinesterase (Scheiber et al., 2014). Inhibition occurs in vitro, whereas it occurs in vivo in biomarkers. Table 1 addresses the assessment of ChEs in various organisms in previous studies (Sabullah et al., 2015b).

Table 1: List of ChE from various sources as biomarker candidate for ecotoxicology monitoring

Enzyme	Species sources	Compound	Related references
Cholinesterase	*Tilapia mossambica*	Sewage water	Al-Ghais, 2013
	Electrophorus electricus	Cu, Ag, Hg	Shukor et al., 2013
	Periophtalmodon schlosseri	Cu, Ag, Hg, As	Sabullah et al., 2013
	Human serum	Mercury chloride	Mahmod et al., 2001
	Daphnia magna	Zn	Diamantino et al., 2003
	Procamborus clarkii	Cd, Pb, Hg	Devi and Fingerman, 1995
	Channa striatus	Hg, Cd, Pb, Ni, Zn	Mat-Jais and Mohamed, 2000

Metal ions including Hg^{2+} interact with functional sulfhydryl group of several enzymes to inhibit their activities. AChE is included among these enzymes even there is no free sensitive sulfhydryl group is observed in its structure. Most of these enzymes present such groups in the form of disulphide bonds or one in an only position accessible through the solution, but unable to react with thiol agents (Aroujo et al., 2016; Frasco, 2007). Unlike pesticides, some heavy metals react as a cofactor for the enzyme, which facilitates the specific substrate to the active site of enzyme (Glusker et al., 1999). However, several heavy metals are also known to cause adverse effect to the organism. The mechanisms of inhibition by heavy metals is versatile to reacts at both active site as well as capable to bind at the external site, which transform the globular shape of enzyme and block the metabolism of substrate (Jomova, Baros & Valko, 2012; Glusker et al., 1999). Heavy metals cannot be degraded by environmental factors and can be only converted to less toxic forms to biological system. For example, mercury in its ionic form, Hg^{+} is very toxic and highly risky to health. However, if this ion reacts with sulphur to form mercury sulphate, it will become less toxic due to its insolubility characteristic in water. The sensitivity of ChE towards toxicant vary according to species to metabolise a single xenobiotic compounds either in vivo or in vitro (Song, Vijyer, Peijnenburg, Galloway & Tyler, 2015) due to variability of behaviour, histology and physiology.

Conclusion

New discovery on the sensitivity of ChE to several classes of contaminants other than organophosphate and carbamate compounds needs to be taken into account for the proper application of this biomarker in environmental pollution preliminary screening programme especially in aquatic environment. ChE extracted from different organs of the same fish species or from different species possess different sensitivity towards anticholinesterastic agents exposed to it. This would make the extracted ChE as a specific biomarker and biosensor for each possible toxicant. In fact, mixed exposures are often to be observed in most cases. It is also worth perceiving that not only diverse composites are able to achieve significant level of anticholinesterase effect, the combination of

different chemical classes can also exert additive or synergistic inhibitory effect on ChE activity. This therefore suggests the need to reconsider the role of ChE in biomonitoring and risk assessment in areas contaminated by several classes of pollutants. In these cases, the use of this biomarker could be that of providing an integrative measurement of the overall neurotoxic risk posed by the whole burden of bioavailable contaminants present in the environment.

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