DEVELOPMENT OF A NOVEL MINI PEPTIDE-BASED BIORECEPTOR FOR HALOALKANE DETECTION

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FBSB 2018 61

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

October 2018

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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October 2018

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Faculty : Biotechnology and Biomolecular Sciences

Haloalkanes are the reactants in pharmaceutical manufacturing, which can be found in the final active pharmaceutical ingredients (APIs) and in the waste as impurities. Due to their toxicity effect to organisms' health, the concern towards haloalkanes is increased. The conventional detection method is time-consuming, costly, lab-based, difficult to operate and not practical for continuous monitoring. Thus, this increases the demand for a simple and rapid device for direct detection of the compounds. Haloalkane dehalogenase (HLD) can be used as the specific bioreceptor to detect the presence of haloalkanes. But, the uses of native HLD are less efficient at extreme condition. Therefore, this study aims to develop a mini protein of HLD as an alternative bioreceptor focusing on sensitivity and stability. A novel mini peptide-based bioreceptor based on HLD from *Xanthobacter autotrophicus* (PDB ID: 2DHC) as template was developed for haloalkane biosensor. Yet Another Scientific Artificial Reality Application (YASARA) software was utilized to create the mini proteins by downsizing approach. Residues were removed gradually to obtain the mini protein while retaining the three active site residues; Asp-124 (nucleophile), His-289 (base), Asp-260 (acid) and two halide stabilizing residues; Trp-125, Trp-175. Five mini proteins comprising 283 amino acids or less, with the highest binding energy (enzyme-substrate complex) and distance of less than 4 Å between Asp-124 and three haloalkanes were chosen as the best validated designs. The recombinant mini proteins were constructed using pET vector and *Escherichia coli* BL21 (DE3) as the expression vector and host, respectively. The smallest mini protein, with 86 amino acids (model 5) was chosen for His-tag affinity purification and subsequent analysis as it could be expressed in soluble form. No catalytic activity was detected with haloalkane substrate. Isothermal titration calorimetry (ITC) showed there was binding interaction between the mini protein and haloalkane. Thermal stability study with circular dichroism (CD) had proven the mini protein possessed higher T_m value at 83.73 °C than the native HLD at 43.97 °C. Optical sensor with tapered multimode glass fiber (TMMF) was fabricated. Protein was immobilized on TMMF with the action of

aminopropyl triethoxysilane (APTES) and glutaraldehyde (GA). The interaction of haloalkane and the immobilized mini protein showed an increment of the UV absorption at 325 nm. Optical sensor proved that the mini protein could act as a potential bioreceptor. However, it demonstrated low sensitivity for haloalkane at 0.0002 μM^{-1} (R^2: 0.9832) with limit of detection (LOD) at 80 µM and low stability. Thus, screen-printed carbon electrodes (SPEs) was used to look for the interaction *via* electrochemical sensor, to enhance the sensitivity and stability. To improve the stability, mini protein structure was mutated with cysteine at residues 49 and 78 to form a disulfide bridge. Bare SPE was modified with gold nanowires coated on the working electrode surface, followed by self-assembly of L-cysteine (Cys) and GA for protein immobilization. The interaction of the mutated mini protein immobilized on SPEs was studied and compared to native HLD immobilized SPEs as positive control. Electrocatalytic oxidation of haloalkane was examined with cyclic voltammetry (CV) and differential pulse voltammetry (DPV) at working potential 0.03 V and -0.1 V, respectively. Electrochemical impedance spectroscopy (EIS) was also performed to detect the binding interaction of haloalkane with the fabricated mutated mini protein. An electrochemical sensor with DPV analysis presented a more sensitive (0.2118 μM^{-1}, R^2: 09741) detection with low LOD at 6 µM. The sensor also demonstrated good repeatability (RSD 4.3%) and reproducibility (RSD 5%) for haloalkane detection. The mutated mini protein based sensor with modified SPEs provided higher sensitivity and better detection of haloalkane than the native HLD. It can be a potential tool in haloalkane detection for immediate application.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN SATU BIORESEPTOR BERASASKAN PEPTIDA YANG NOVEL UNTUK MENGESAN HALOALKANA

Oleh

NURUL HAZWANI BINTI DAUD

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Haloalkana adalah reaktan dalam pembuatan farmaseutikal, yang boleh didapati di dalam bahan farmaseutikal aktif akhir (API) dan sisa buangan sebagai kekotoran. Disebabkan oleh kesan toksiknya terhadap kesihatan organisma, kebimbangan terhadap haloalkana semakin meningkat. Kaedah pengesanan konvensional adalah memakan masa, mahal, berdasarkan makmal, sukar untuk dikendalikan dan tidak praktikal untuk pemantauan berterusan. Oleh itu, ini meningkatkan permintaan untuk peranti yang mudah dan cepat untuk mengesan sebatian secara langsung. Haloalkana dehalogenase (HLD) boleh digunakan sebagai bioreseptor yang spesifik untuk mengesan kehadiran haloalkana. Tetapi, penggunaan HLD asli kurang cekap pada keadaan yang melampau. Oleh itu, kajian ini bertujuan untuk membangunkan protein mini HLD sebagai bioreseptor alternatif yang tertumpu kepada kepekaan dan kestabilan. Bioreseptor yang novel berasaskan mini peptida telah dibangunkan untuk biosensor haloalkana berpandukan kepada HLD daripada *Xanthobacter autotrophicus* (PDB ID: 2DHC) sebagai acuan. Sensor ini memberi tumpuan kepada kepekaan dan kestabilan. Perisian YASARA telah digunakan untuk mencipta protein mini dengan pendekatan pengurangan saiz. Residu dikeluarkan secara beransur-ansur untuk mendapatkan protein mini sambil mengekalkan tiga residu tapak aktif; Asp-124 (nukleofile), His-289 (asas), Asp-260 (asid) dan dua residu penstabil halida; Trp-125, Trp-175. Lima protein mini terdiri daripada 283 asid amino atau kurang, dengan tenaga mengikat tertinggi (kompleks enzim-substrat) dan jarak kurang dari 4 Å antara Asp-124 dan tiga haloalkana dipilih sebagai reka bentuk yang terbaik untuk disahkan. Protein rekombinan mini dibina menggunakan vektor pET dan *Escherichia coli* BL21 (DE3) masing-masing sebagai vektor ekspresi dan hos. Protein mini terkecil dengan 86 asid amino (model 5) telah dipilih untuk proses penulenan dan analisis seterusnya kerana dapat mengekspresskan protein yang larut. Tiada katalitik aktiviti dikesan dengan substrat haloalkana. Isotermal kalorimetri penitratan (ITC) menunjukkan terdapat interaksi mengikat diantara protein mini dan haloalkana. Kajian kestabilan termal dengan dichroism bulat (CD) telah membuktikan bahawa protein mini

mempunyai nilai T_m yang lebih tinggi pada 83.73 ° C berbanding HLD asli pada 43.97 ° C. Optik sensor dengan gentian kaca pelbagai mode tirus (TMMF) telah dibina. Protein telah dipegunkan di atas TMMF oleh aminopropyl triethoxysilane (APTES) dan glutaraldehid (GA). Interaksi haloalkana dan protein mini yang pegun menunjukkan peningkatan penyerapan UV pada 325 nm. Sensor membuktikan bahawa protein mini yang direka berpotensi sebagai bioreseptor. Walaubagaimanapun, ia menunjukkan kepekaan yang rendah untuk mengesan haloalkana pada 0.0002 μM^{-1} (R^2: 0.9832) dengan had pengesanan (LOD) pada 80 µM dan kestabilan yang rendah. Oleh itu, elektrod karbon bercetak skrin (SPE) digunakan untuk mencari interaksi melalui sensor elektrokimia, untuk meningkatkan kepekaan dan kestabilan. Untuk meningkatkan kestabilan, struktur protein mini telah dimutasi dengan cysteine pada residu 49 dan 78 untuk membentuk satu jambatan disulfida. SPE kosong telah diubahsuai dengan nanowayar emas yang dilapisi pada permukaan elektrod kerja, diikuti oleh penyusunan secara sendiri L-cysteine (Cys) dan GA untuk pemegunan protein. Interaksi protein mini bermutasi di SPE telah dikaji dan dibandingkan dengan HLD asli yang pegun pada SPE sebagai kawalan positif. Pengoksidaan elektrokatalitik haloalkana telah diperiksa dengan voltammetrik kitaran (CV) dan voltammetrik denyutan nadi (DPV) pada keupayaan kerja masing-masing 0.03 V dan -0.1 V. Spektroskopi impedans elektrokimia (EIS) juga dijalankan untuk mengesan interaksi mengikat haloalkana dengan protein mini yang dimutasi. Sensor elektrokimia dengan analisis DPV menunjukkan pengesanan yang lebih peka (0.2118 μM^{-1}, R^2: 09741) dengan LOD pada 6 µM. Sensor juga menunjukkan pengulangan (RSD 4.3%) dan kebolehulangan (RSD 5%) yang baik untuk pengesanan haloalkana. Sensor berasaskan protein mini yang dimutasi dengan SPE yang telah diubah suai memberikan kepekaan yang lebih tinggi dan pengesanan haloalkana yang lebih baik daripada HLD asli. Ia boleh menjadi alat yang berpotensi dalam mengesan haloalkana untuk aplikasi segera.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest appreciation and gratitude to my supervisor, Prof. Dato' Dr. Abu Bakar Salleh for his supervision throughout this project. I would like to thank him for his valuable advice, support and guidance. Besides that, an appreciation also dedicated to my co-supervisor, Assoc. Prof. Dr. Adam Leow Thean Chor, for his valuable feedback and Dr. Siti Nurbaya Oslan for her valuable suggestions. I'm also would like to thank Dr. Muhammad Hafiz Abu Bakar for his kind advice on optical sensor and comments on improving my thesis. Sincere thanks to all EMTECH laboratory colleagues especially, Ang Thiau Fu, Jonathan, Fatin Liyana, Diyanah and Shazwani.

A special thanks also goes to my beloved family. Thanks for the moral support encouragement and understanding given to me especially by my beloved husband, Muhammad Rizal Rosli throughout the entire period of my project. These words are not enough to describe my gratitude and appreciation for everyone's help. Thanks all!

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

aa	Amino acid
Å	Angstrom
$Å^2$	Angstrom square
APTES	aminopropyl triethoxysilane
bp	base pair
CV	Cyclic voltammetry
Da	Dalton
DPV	Differential pulse voltammetry
eV	Electronvolt
g	Gram
h	Hour
HLD	Haloalkane dehalogenase
IPTG	isopropyl-β-D-tiogalactopyranoside
K	Kelvin
kDa	KiloDalton
K_m	Michaelis-Menten constant
L	Liter
LOD	Limit of detection
LOQ	Limit of quantification
MD	Molecular dynamics
M	Molar
mg	Milligram
ml	Milliliter
min	Minute

mV	Milivolt
mVs^{-1}	Milivolt per second
NWs	Nanowires
nm	Nanometer
nmol	Nanomole
PCR	Polymerase chain reaction
pH	Exponential of the concentration of hydrogen ion
pmol	Picomole
Rpm	Rotation per minute
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
RSD%	Relative standard deviation percentage
s	Seconds
SASA	Solvent accessible surface area
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sp.	Species
SPEs	Screen-printed electrodes
TMMF	Tapered multimode fiber
U	Unit of enzyme activity
U/ml	Unit per milliliter
U/mg	Unit per milligram
UV	Ultra violet

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Genotoxic impurities (GTIs) in the final active pharmaceutical ingredients (APIs) gain attention and become a serious matter of concern in the present days for all pharmaceutical industries. Hence, the need to investigate the genotoxic level in pharmaceutical manufacturing is very crucial due to the usage of dangerous compounds. Haloalkane is a toxic compound and used as a reactant for alkylation in drug synthesis and utilized as organic solvents in the drug product manufacturing process (Szekely *et al*., 2012). Over the years, regulatory health agencies have consistently expanded those level of security required to control GTIs in drug substance materials, thus raising the bar on prerequisites to quantify haloalkane towards low ppm levels. Besides that, pharmaceutical industries generate a huge quantity of wastes during manufacturing and maintenance operations. Improper disposable of pharmaceutical waste products is also an issue in the pharmaceutical industry (Patneedi and Prasadu, 2015). Additionally, several types of pharmaceuticals compounds have been detected in water samples from ng/l to µg/l range (Kapoor, 2015). Thus, a very sensitive analysis needs to be performed to monitor and quantify the amounts of genotoxic impurities in pharmaceutical products and in the environment.

Conventional analytical techniques like LCMS/MS and GC-MS/MS based on physicochemical properties of the compounds are employed towards the detection of impurities. These techniques are highly selective and sensitive; however, they are impractical for long-term continuous monitoring analysis, time-consuming, expensive and laborious (Paul and Paul, 2015). Owing to that, a biosensor can be an alternative tool to the conventional technique. Biosensor is a detection system composed of a bioreceptor and a transducer. Catalytic biosensor is usually used for monitoring haloalkane compounds, utilizing purified enzyme as the bioreceptors. It incorporates with the purified enzyme to provide high selectivity and has the potential of modifying catalytic properties *via* genetic engineering (Rogers, 2006). The first biosensor exploiting a purified haloalkane dehalogenase (HLD) as the bioreceptor for haloalkane detection (Bidmanova *et al*., 2010). However, the developed sensor demonstrated low sensitivity towards haloalkane (Bidmanova *et al*., 2010).

Therefore, the main focuses of this study are to develop a biosensor that is specific, sensitive and stable for continuous mode of haloalkane detection. HLD was selected as a specific bioreceptor due to its ability to catalyze specifically the conversion of haloalkane to alcohol, halides, and proton (Bidmanova *et al*., 2013). Immobilization of the bioreceptor on the transducer is a necessary and critical step in designing biosensors, where it strongly affects the analytical performance (Andresscu and Sadik, 2004). The suitable immobilization strategies need to preserve the active site that

essential for enhancement of their catalytic properties (Koudelakova *et al*., 2013). The immobilized enzymes are more robust and more resistant to environmental changes than the free enzymes, where they allow multiple uses of the enzymes and continuous operation of enzymatic processes (Homaei *et al*., 2013).

Optical and electrochemical sensors are the most widely used tools in the development of catalytic biosensor (Rodriguez-Mozaz *et al*., 2004). Both sensors are also reported for the detection of dehalogenase reaction product (Peter *et al*., 1996). Optical sensor with tapered multimode fiber (TMMF) has proven to be a viable technology for sensing applications (Qiu *et al*., 2016). In an electrochemical sensor, screen-printed electrodes (SPEs) are commonly used as electrodes due to the moderate cost, highly reproducible and reliable sensors. Additionally, various chemical modifications on the working surface SPEs can be performed (Bergamini *et al*., 2007). Nanomaterials like nanowires, increase the specific surface area for the electrochemical reaction, subsequently improve sensitivity and decrease the limit of detection (LOD) (Swierczewska *et al*., 2013).

Current developed sensor for haloalkane monitoring with native HLD loses its stability at a higher temperature (above 50 °C) due to structure denaturation (Jesenská *et al*., 2002). Thus, this increases the demand for creating mini proteins which are stable as bioreceptors and suitable for harsh industrial application. Mini protein is a polypeptide which nevertheless exhibits a well-defined tertiary structure (Wang *et al*., 2008). It provides a simple and useful model system for studying the native structure and also knowledge in understanding the relationship between protein structure and function (Polticelli *et al*., 2001). Through this study, the computational approach was used to design mini proteins from the 3D structure of native HLD from *Xanthobacter autotrophicus* (PDB ID: 2DHC) which acted as a template. The application of powerful computational approach for functional novel protein designing has recently succeeded in engineering target activities (Tiwari *et al*., 2012).

1.2 Problem statements

The level of GTIs needs to be monitored due to the utilization of toxic compounds such as in the manufactured pharmaceutical product and waste. A high amount of GTIs, exceeding a certain acceptable limit has a various negative effect on human health. Acceptable limits for haloalkane GTIs in pharmaceutical product is at 1.5 ppm (1500 µg L^{-1}) for 1 g daily dose of product. Meanwhile, lower acceptable limit of haloalkane set in drinking water is at 0.05 µg L^{-1} to 5 µg L^{-1}. Normally, gas chromatography coupled with mass spectroscopy is used for the analysis of haloalkane compounds. However, these methods are time-consuming, costly and require the use of highly skilled personnel. Due to that, demand for rapid, low cost, and *in-situ* methods of detecting haloalkane is increasing. Enzyme is considered as a bioreceptor and very specific to detect the substrates (contaminant). However, enzymes are large biomolecules that susceptible to denaturation at extreme conditions. Fabrication and operation of native HLD as bioreceptor are more costly than the mini protein. By incorporating a mini protein as an alternative bioreceptor may solve the problem. Mini

protein is less bulky and should have the advantage in term of stability as compared to the native HLD.

1.3 Significance of the study

This work has presented the first optical and electrochemical-based sensor utilizing mini proteins as bioreceptors. The mini protein may provide stability at the same time provide higher sensitivity and selectivity. In the long run, the newly developed sensor is economy scale.

1.4 Objectives

The main objective of this study is to develop a novel mini peptide-based bioreceptor for the detection of haloalkane compounds. Listed below are the specific objectives of this study:

- To design a mini protein of haloalkane dehalogenase (HLD).
- To immobilize the designed mini protein with the transducer based on optical and electrochemical techniques.
- To evaluate the performance of the developed biosensor by using optical and electrochemical techniques.

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