UNIVERSITI PUTRA MALAYSIA

DESIGN OF MINI PROTEIN THAT MIMICS URICASE IN THE PRELIMINARY DEVELOPMENT OF NANOWIRE-BASED URIC ACID BIOSENSOR

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

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Chairman : Professor Dato' Abu Bakar Salleh, PhD
Faculty : Biotechnology and Biomolecular Sciences

Mini protein is used as an alternative bioreceptor to native enzyme, which is costly and less stable. Mini protein that mimics enzyme may be utilized as the bioreceptor for the determination of metabolite in biological fluids. In this study, a novel uric acid biosensor of mini protein immobilized onto screen printed carbon electrodes (SPEs) was developed for uric acid detection to replace conventional method. Uricase is a large tetrameric protein carrying two active sites in each pair of dimer. This generates an interest for a mini protein to function as bioreceptor that may replace the large native enzymes. Five mini proteins comprising 20, 40, 60, 80 and 100 amino acids were designed based on the conserved active site residues within the same dimer, using the 2yzb template. Utilizing Yet Another Scientific Artificial Reality Application (YASARA) software, four target structures were predicted for each mini protein model, by multistep process of homology modeling. Then the stereochemical quality of the structure model was verified by PROCHECK and ERRAT programs. The best evaluated structures (model 3 of mp20, model 2 of mp40, model 3 of mp60, model 3 of mp80 and model 2 of mp100) were simulated with molecular dynamics (MD) simulations using YASARA, to study protein stability and folding. Five mini proteins with the highest binding energy (enzyme-substrate complex) from docking were chosen in MD simulation analysis (mini protein and the substrate). The results also proved that the present of substrate in the protein structure helped to improve protein folding. Five recombinants of mini proteins (mp20, mp40, mp60, mp80 and mp100) were constructed in pET32a vector and all of them were successfully expressed into *E. coli* Bl21 (DE3). The smallest mini protein with 20 amino acids (mp20) was chosen for His-tag affinity purification. The purified mp20 showed no activity in uricase assay. Subsequently, the approach was to look for binding affinity of mini protein and substrate (uric acid) via Isothermal titration calorimetry (ITC) and circular dichroism (CD) spectra. The ITC and CD results had proven that there was binding interaction between uric acid and the mini protein structure. A disposable uric acid biosensor on modified gold nanowires screen printed carbon electrode (SPEs) was fabricated. The working surface of the SPEs electrode was modified by gold nanowires deposited on the surface, followed by self-assembly of L-cysteine and glutaraldehyde. The mini protein immobilized SPEs was studied and compared to uricase immobilized SPEs as

positive control. The electrocatalytic oxidation of uric acid was examined using cyclic voltammetry (CV) of a range between -1.0 and 1.0 V, at working potential 0.4 V (50 mVs^{-1} scan rate). The sensor demonstrated that by using mini protein as the bioreceptor, the nanowire biosensor exhibited similar stability, sensitivity, selectivity, good reproducibility and repeatability for uric acid determination, with a linear range from 0.01 mM to 1.0 mM and a detection limit of 0.1 mM as compared to control (uricase as the bioreceptor). Interestingly, the mini protein immobilized SPEs had strong affinity for uric acid compared to uricase immobilized SPEs with small value of the apparent Michaelis-Menten Constant (K_M^{app}). Besides, The mini protein immobilized SPEs had similar performance as compared to commercial uric acid biosensor in the market. In conclusion, the developed mini protein immobilized SPEs can be considered as a useful tool to replace conventional method in uric acid detection.

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

MEREKA PROTEIN MINI MENYERUPAI URIKASE DALAM PEMBANGUNAN AWAL BIOSENSOR ASID URIK BERASASKAN WAYARNANO

Oleh

ARILLA SRI MASAYU BINTI ABD RAHIM

Mei 2017

Pengerusi: Profesor Dato' Abu Bakar Salleh, PhD
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Protein mini merupakan satu alternatif bioreseptor menggantikan enzim natif yang merupakan mahal dan kurang stabil. Protein mini yang meniru enzim telah digunakan sebagai bioreseptor bagi penentuan metabolit di dalam cecair biologi. Di dalam kajian ini, biosensor asid urik yang novel dengan menggunakan protein mini di atas skrin bercetak elektrod karbon (SPEs) telah dibangunkan untuk mengesan asid urik di mana ia menggantikan kaedah konvensional. Urikase adalah protein tetramerik besar yang membawa dua poket dalam setiap pasangan dimer. Hal ini telah menyebabkan permintaan yang tinggi untuk menggantikan protein mini yang berfungsi sebagai bioreseptor untuk pengesanan metabolit yang boleh menggantikan konvensional enzim asli yang besar dan tidak stabil. Lima protein mini yang terdiri daripada 20, 40, 60, 80 dan 100 asid amino telah direka berdasarkan tapak sisa aktif dipelihara dalam dimer yang sama dengan menggunakan templat 2yzb. Empat struktur sasaran telah diramalkan untuk setiap model protein mini dari proses berperingkat homologi pemodelan daripada perisian YASARA dan mengesahkan kualiti stereokimia model protein struktur dengan menggunakan PROCHECK dan ERRAT. Struktur terbaik setiap protein mini iaitu; model 2 dari mp20, model 2 dari mp40, model 3 dari mp60, model 3 dari mp80 dan model 2 dari mp100 telah dipilih untuk disimulasikan dengan dinamik molekul (MD) simulasi menggunakan YASARA untuk mengkaji lipatan protein. Tahap kestabilan tenaga pengikat yang tinggi dalam model mini protein dengan substrat (enzim-substrat kompleks) telah dipilih dalam analisis simulasi MD. Keputusan telah menunjukkan kehadiran substrat dalam struktur protein telah membantu meningkatkan pelipatan protein. Untuk mengesahkan keputusan simulasi MD, lima rekombinan protein mini (mp20, mp40, mp60, mp80 dan mp100) telah dibina bersama vektor pET32a. Semua rekombinan protein mini telah berjaya diekspresskan ke dalam *E.coli* BL21 (DE3). Protein mini terkecil yang mengandungi 20 asid amino (mp20) telah dipilih untuk proses penulenan melalui penulenan His-tag. Namun begitu, protein mini tulen tidak menunjukkan aktiviti di dalam assai urikase. Oleh itu, pendekatan dalam mengkaji pertalian pengikatan di antara mini protein dan asid urik melalui Isotermal kalorimeter pentitratan (ITC) dan dichroism bulat (CD) spektrum telah dijalankan. Keputusan ITC dan CD menunjukkan terdapat interaksi pengikatan di antara asid urik dan struktur protein mini. Biosensor asid urik menggunakan nanowayar emas di atas skrin karbon dicetak elektrod (SPE) telah bina.

SPE elektrod telah diubahsuai dengan mendepositkan nanowayar emas ke atas permukaan SPE diikuti oleh pendepositan secara sendiri oleh L-cysteine dan glutaraldehyde. Dalam kajian ini, protein mini atas SPE telah dkaji and dibandingkan dengan urikase atas SPE yang merupakan kawalan positif. Pengoksidaan electrokatalitik asid urik telah disiasat menggunakan voltammetri berkitar (CV) di antara -1.0 hingga 1.0 V yang pada bekerja pada 0.4 V (50 mVs^{-1} kadar imbasan). Sensor menunjukkan dengan menggunakan protein mini sebagai bioreseptor, menunjukkan persamaan dalam kestabilan, kepekaan, pemilihan dan kebolehulangan untuk menentukan asid urik dari 0.01 mM kepada 1.0 mM di mana had pengesanan adalah 0.1 mM setelah dibandingkan dengan kontrol. Menariknya protein mini atas SPE menunjukkan pertalian yang kuat ke atas asid urik berbanding urikase atas SPE dengan nilai Michaelis-Menten tetap (K_M^{app}) yang kecil. Selain itu, penggunaan protein mini atas SPE turut mempunyai prestasi yang sama apabila dibandingkan dengan biosensor asid urik komersil. Kesimpulannya, pembangunan protein mini atas SPE dianggap alat yang berguna untuk menggantikan kaedah lama dalam pengesanan asid urik.

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ARILLA SRI MASAYU ABD RAHIM

I certify that a Thesis Examination Committee has met on 29 May 2017 to conduct the final examination of Arilla Sri Masayu binti Abd Rahim on her thesis entitled "Design of Mini Protein that Mimics Uricase in the Preliminary Development of Nanowire-Based Uric Acid Biosensor" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment 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
AA	Ascorbic acid
Å	Armstrong
$Å^2$	Armstrong square
bp	Base pair
cm	Centimeter
CV	Cyclic voltammetry
Da	Dalton
DNA	Deoxynucleic acid
eV	Electronvolt
g	Gram
h	Hour
H_2O_2	Hydrogen peroxide
IPTG	Isopropyl-β-D-tiogalactopyranoside
K	Kelvin
kDa	Kilo Dalton
K_M	Michaelis-Menten constant
K_M^{app}	Apparent Michaelis constant
L	Liter
LOD	Limit of detection
MD	Molecular dynamics
M	Molar
mg	Milligram
ml	Milliliter
min	Min
mp	Mini protein
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
Sp.	Species
SPEs	Screen printed carbon electrode
U	Unit of enzyme activity
U/ml	Unit per milliliter
U/mg	Unit per milligram
UV	Ultra violet
UA	Uric acid

v/v	Volume per volume
V	Voltage
V/s	Voltage per seconds
V_{max}	Maximum velocity
w/v	Weight per volume
µg	Microgram
µl	Microliter
µmol	Micromole
µA	MicroAmpere
3D	Three dimensional
%	Percentage
º	Degree

CHAPTER 1

INTRODUCTION

1.1 Background of study

Nowadays, modern people's lives are intense and busy. The combinations of irregular schedules, unconventional diet, and insufficient rest and exercise lead to gout. It occurs when excess uric acid accumulate in the body and gets deposited in the joints. As the primary end product of purine metabolism, uric acid is present in biological fluids such as blood and urine. Increased amount of uric acid (>7 mg/dl) in these fluids also lead to pathological conditions, such as chronic renal disease, urolithiasis, and nephrolithiasis. Alternative methods such as calorimetry and HPLC are laborious, expensive, time consuming and impractical for long term continuous monitoring analysis (Rawal *et al*., 2012). Thus, the approach of electrochemical uric acid biosensor poses as a simple, rapid and cost effective technique. Enzyme-based electrochemical biosensors have been used widely, such as in health care, food safety, and environmental monitoring, based on the principles of redox reaction. During the immobilization of the bioreceptors, the active site of the enzyme must be preserved and satisfactory electrochemical communicability between the redox site and the electrode (Putzbach and Ronkainen, 2013). In the electrochemical biosensor, screen printed carbon electrode (SPEs) is widely used as the electrode due to its potentialities in aspects of portability, simple instrumental design, and moderate cost. It also allows various chemical modifications on the working surface electrode (Bergamini *et al*., 2007).

With the growing of nanotechnology fields, it has been proven that the utilization of nanomaterials in uric acid biosensor can enhance the electrochemical responses. Nanomaterials such as nanoparticles, nanowires and nanotubes play an important role in the development of biosensors. In this study, gold nanowires have been consistently used as an electrochemical transducer, to increase the surface area of the electrode in order to improve sensitivity and lower Limit of Detection (LODs). Nanowire is defined as semiconducting particles having a high ratio, with diameters of <1 μm and lengths as tens of microns (García *et al*., 2014). Currently, most uric acid biosensor in the market work based on the interaction between uricase and uric acid, to produce redox reaction. However, a recurring disadvantage of the system is due to the susceptibility of uricase to interferences in the blood, and its lesser stability. This generates an increase of demand for mini proteins to function as bioreceptors for metabolite detection. This is apparent especially when it is compared to native enzyme (uricase), to overcome the selectivity and instability issues. Uricase is a tetrameric structure, a large protein with its active site located on the interface within the same dimer with five catalytic residues for binding interactions (Colloc'h *et al*., 2008).

Knowledge of the three-dimensional (3D) structure of uricase is essential to understand the relationship between its structure and function. These 3D structures can be ascertained at high resolution by either computational analysis or experimental methods (X-ray crystallography and Nuclear Magnetic Resonance Spectroscopy (NMR)). Screening uricase from different sources is important to study the structure-

function relationship of a catabolic enzyme. This will provide an insight into the molecular basis of the biological process. 3D structure of uricase has been generated from different sources such as *Aspergillus flavus* (PDB ID 1WS2), *Arthrobacter globiformis* (PDB ID 2YZC) and *Agrobacterium tumefaciens* (PDB ID 2O8I) (Beedkar *et al*., 2012). The computational protein design is currently viewed as a promising tool to address the complexities in generating made-to-order biocatalysts and to solve problems in protein engineering. Additionally, it can also facilitate and enhance the functions of natural proteins, in order to develop novel protein-based systems (Lippow and Tidor, 2007; Kang and Saven, 2007). Various computer programmes such as YASARA software are widely used in the protein design. Besides, by designing novel mini protein binding sites present additional challenges to the protein conformational and folding theory.

1.2 Problem statements

Abnormal uric acid level in biological fluids (blood and urine) is diagnostic for several diseases. Therefore, the demand for routine monitoring uric acid sensor that is simple to use, produces rapid measurements, and is low in cost is increasing. Incorporating enzymes like uricase in electrochemical uric acid biosensors is costly, less stable and generally faces varying degrees of interference such as ascorbic acid, which can be oxidized at a potential close to the uric acid. To overcome this issue, use of mini protein that mimics uricase as an alternative bioreceptor to replace the native enzyme (uricase) is highly suggested as a promising way to solve this problem. The tetrameric structure of uricase is derived from the orientations of five catalytic residues in the active site. By using the computer program, mini protein was designed to mimic the uricase active site. This is apparent especially when it is compared to native enzyme, to overcome the selectivity issue.

1.3 Significance of study

The fabrication of gold nanowires on screen printed electrode (SPEs) is the first being employed in the uric acid electrochemical biosensor with mini protein with 20 amino acids as bioreceptor. The mini protein that mimics uricase was designed with the characteristics of reproducing the enzyme's functionality as the alternative to uricase, which were costly and less stable. Moreover, designing mini proteins could improve the insight regarding the relationship between protein structure and function. In addition, no extensive study has been done to design a novel mini protein from a native structure possessing an active site in the interface of its two monomers. This will prove to be challenging and yet fruitful. This innovative uric acid nanowire biosensor with immobilized mini protein will improve the sensor's performance, especially in the aspects of its stability against interferences such as ascorbic acid as the interference compound in blood serum.

1.4 Objectives

The goal of this study is to develop novel and simple uric acid electrochemical biosensor based on gold nanowires modified SPEs using mini protein as bioreceptor. The following specific objectives were designed to achieve this goal;

I. To design a mini protein that mimics uricase
II. To integrate the designed mini protein with nanowire transducer
III. To optimize and characterize biosensor

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