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CONFORMATIONAL DESIGN AND CHARACTERIZATION OF A TRUNCATED DIAMINE OXIDASE

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfiment of the requirement for the degree of Master of Science

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Diamine oxidase, DAO (EC 1.4.3.22) is an oxidoreductase enzyme that degrades primary amines to produce aldehyde, H_2O_2 and NH_3. DAO, also known as histaminase, is a homodimer protein consisting of 60 to 105 kDa subunits. Being a large protein, DAO is more susceptible to destabilising agents such as heat and pH, similar to other large proteins. Due to the large structure of native diamine oxidase, researchers are being actively conducted to develop a more versatile, and stable protein because big molecules do not perform well in microsystem. It also gave more flexiblity and low cost in production. Thus, the aim of this study was to develop a low cost miniature version of the native protein while mimicking its catalytic function via protein engineering and biomolecular approaches.

In the protein engineering approach, a rational design was applied to produce a mini protein that can offer more potential i.e. for use in the bioinformatics and biological discoveries. The gene encoding diamine oxidase was identified in the UNITPROT/KB database and it showed a 61% sequence similarity when blasted with NCBI database. The protein structure was built using a homology modeling in YASARA™ . All the constituent functional domains of the protein were prescisely defined and shown as predicted. The miniaturization process of the protein was started with size reduction after its conserved domain has been predicted. Based on several observations, there were three domains in the predicted structure with each domain having its own major contribution to the protein structure. The domain 4 that contained the active site was selected for the splicing process in order to retain the enzyme's active site. The molecular dynamic simulation study was executed within 20 ns for each of the structures. This step is important to detect any abnormality in the *in silico* structure. Based on results from the RMSD, SASA and RMSF it has been determined that the mini

DAO has maintained its stability which is in alignment with the parental protein. The model protein was then submitted to a protein-ligand docking simulation to study the interaction between the enzyme and a substrate. The ligand interaction showed that histamine and spermidine were excellent substrates for the mini protein because they maintained the preferences of the native protein, which often chooses short-chain length substrates. Quality assessments of the native and mini protein were also conducted using several types of verification software.

Subsequently, a number of model structures were generated using a biomolecular method where the native and mini protein has been successfully cloned into vector pET102/TOPO during the first stage. The transformation of the recombinant proteins was also conducted as the sequencing result was identical to the expected reference sequence. Next, the recombinant cell was expressed into *E. coli* BL21 (DE3) and induced with 20% of IPTG, at 25°C for 24 h. A final mini protein activity of 62.283 U/mL was determined. Crude enzyme obtained from the intracellular expression was then purified. Purification strategy for obtaining fusion mini protein with his-tagged was established. Furthermore, fusion native purification was conducted to recover the fusion protein through affinity chromatography Ni-Sepharose. High purification yield of 88% was obtained for mini protein fusion tagged (His-tagged + mature mini protein). The molecular weight of the mini protein was 42.2 kDa by SDS-PAGE. As expected, the mini protein showed a high level of interaction with histamine. Surprisingly, the mini protein had also interacted with spermidine, a long side-chain ligand, which showed that its specificity towards different substrates had broadened up. By maintaining the native protein's pH profile, the mini protein was highly active at 40 °C and stable at pH 7, with a half-life of 80 min at 50 °C. Results from the kinetic studies showed that the K_m value for native diamine oxidase was 0.274 mM and 1.3 mM for the mini protein. Based on these results, it can be concluded that the mini protein was more active than the native protein but it has kept the same interaction with histamine. This characteristic has been identified by purely rational as well as experimental combinatorial design techniques. The results have been significant to postulate that the mini protein was more stable and versatile compared to the native protein due to its size, thermostability and broad substrate specificity. This mini protein is a suitable candidate for industrial biocatalyst and biological sensor applications.

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Diamina oksida, DAO (EC 1.4.3.22) adalah kumpulan enzim oksidoreduktase yang menguraikan kumpulan utama amina untuk menghasilkan aldehid, H_2O_2 dan NH_3. DAO juga dikenali sebagai 'histaminase' yang merupakan satu protin bersubunit sama yang mempunyai saiz 60 hingga 105 kDa untuk setiap subunit. Oleh kerana ianya protein yang besar, DAO adalah terdedah kepada ejen-ejen yang menggugat kestabilan seperti haba dan pH, sama seperti protin besar yang lain. Oleh kerana diamina oksida asal mempunyai struktur yang besar, penyelidikan giat dilaksanakan untuk membangunkan protin yang lebih serba boleh, fleksibel dan stabil kerana pada kebiasaannya molekul-molekul yang besar tidak menunjukkan prestasi baik dalam sistem mikro. Oleh itu, kajian ini bertujuan untuk menghasilkan versi mini protin asal yang berkos rendah sambil berupaya meniru fungsi pemangkin melalui pendekatan kejuruteraan dan biomolekul protin.

Dalam pendekatan kejuruteraan protin, reka-bentuk rasional yang digunakan untuk menghasilkan protin mini boleh menawarkan banyak potensi seperti dalam kegunaan bioinformatik dan penemuan biologi. Gen pengekodan diamina oksida dikenal pasti di dalam pangkalan data UNITPROT/KB dan ia menunjukkan 61% persamaan turutan apabila disuaigandingkan terhadap pangkalan data NCBI. Struktur protin ini dibina menggunakan teknik homologi permodelan di YASARA™. Semua juzuk fungsian domain protin yang tepat telah ditentukan dan ditunjukkan seperti apa yang diramalkan. Proses pengecilan protin bermula dengan pengurangan saiz protin selepas pemuliharan domain dijalankan. Berdasarkan beberapa pemerhatian, terdapat tiga domain di dalam struktur setiap domain mempunyai sumbangan utama tersendiri di dalam struktur protein. Domain ke-4 yang mengandungi tapak aktif dipilih untuk melalui proses pengecilan bagi mengekalkan tapak aktif enzim yang sedia ada. Kajian simulasi dinamik

molekul ini telah disempurnakan dalam tempoh 20 ns bagi setiap struktur. Ini adalah penting untuk mengesan kecacatan struktur secara komputer. Berdasarkan keputusan dari RMSD, SASA dan RMSF, bagi menentukan bahawa mini protin telah mengekalkan kestabilan yang sejajar dengan protin asal. Model-model protin ini kemudiannya menjalani simulasi interaksi antara subtrat dan ligan bagi mengenalpasti kecenderungan enzim tersebut dengan suatu substrat. Interaksi ligand ini mendapati histamina dan spermidina merupakan substrat yang terbaik untuk protin mini kerana ia mengekalkan ciri-ciri asal protin asli. Penilain kualiti bagi setiap protin juga dijalankan dengan menggunakan beberapa jenis perisian pengesahan.

Seterusnya, sebagai peringkat permulaan, beberapa struktur model telah dihasikan dengan menggunakan pendekatan biomolekul, di mana, protin asli dan mini protin telah berjaya diklonkan ke dalam vektor pET102/TOPO. Transformasi rekombinan protin ini juga telah menunjukkan 100% jujukan penjajaran terhadap jujukan rujukan. Kemudian, sel rekombinan protin mini tersebut telah berjaya diekpresikan di dalam *E.coli* BL21(DE3) dengan 20% IPTG pada suhu 25 °C selama 24 j. Sebanyak 62.283 U/mL telah dikenalpasti sebagi aktiviti protin mini. Enzim mentah yang telah diperolehi daripada ekspresi intrasel, kemudiannya ditulenkan. Pemurnian strategi untuk mendapatkan protin mini yang bergabung dengan tag-his telah dijalankan melalui kromatografi Ni-Sepharose. Hasil penulenan yang tinggi sebanyak 88% telah diperolehi daripada protin mini gabungan (Tag-his + protin mini yang matang). Molekul protin mini ini bersaiz adalah 42.2 kDa seperti yang ditunjukkan pada SDS-PAGE. Seperti jangkaan, protin mini telah menunjukkan tahap interaksi dengan histamina yang tinggi. Menariknya, mini DAO juga telah menunjukan interaksi yang bagus terhadap spemidina, sebuah ligan yang menpunyai rantaian luar yang panjang. Ini menunjukan, spesifisiti substrat telah diperluaskan. Dengan mengekalkan profil pH seperti protin asal, protin mini ini didapati sangat aktif pada 40 °C dan stabil pada pH 7, dengan nilai separuh hayat selama 80 min pada 50 °C. Manakala, hasil daripada kajian kinetic, nilai K_m protin asal adalah 0.274 mM dan 1.3 mM bagi protin mini. Ciri-ciri ini telah disokong melalui eksperimen kaedah rasional. Berdasarkan pemerhatian ini, maka disimpulakn bahawa protin mini ini lebih stabil dan serba boleh jika dibandingkan dengan protein asal berikutan saiz, tahap ketahanan suhu dan perluasan substrat. Ini menjadikan protin mini ini sesuai untuk aplikasi dalam pelbagai bidang industri serta aplikasi penderiaan biologi.

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

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LIST OF ABBREVIATIONS

µL	Microliter
µm	Micrometer
AGAO	*Arthrobacter globiformis* amine oxidase
BA	Biogenic amine
BSAO	*Bovine serum* amine oxidase
CAO	Copper amine oxidase
Å	Amstrong
C	Celcius
cm	Centimeter
Co	Cobalt
Da	Dalton
DH2O	Distilled water
DNA	Deoxyribonucleic acid
ECAO	*Escherichia coli* amine oxidase
g	Gram
g/L	Gram per liter
h	Hour
hDAO	Human diamine oxidase
hVAP	Human vascular adhesion protein
HPAO	*Hansenula polymorpha* amine oxidase

IPTG	Isopropyl ß-D Thiogalactoside
kbp	kilo base pair
kDa	kilo Dalton
LB	Luria bertani
mg	miligram
ml	mililiter
MAO	Mono amine oxidase
M	Molar
MD	Molecular Dynamic
nm	nanometer
Ni	Nickel
PEM	Particle Mesh Ewald
RAO	Retina Amine Oxidase
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
OD	Optical Density
PCR	Polymerase Chain Reaction
SASA	Solvent Accessible Surface Area
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SOC	Super Optimum Broth
TEMED	N, N, N, N-Tetramethyllenediamine
TPQ	Topaquinone

U/mg	Unit per miligram
U/mL	Unit per mililiter
UV	Ultraviolet
v/v	Volume per volume
V	Volt
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

An enzyme's function is greatly influenced by its amino acid composition and sequence leading to protein conformation. Uniquely, an enzyme can catalyse a specific substrate due to the clefts on its surface, known as active sites that are complimentary in shape to that substrate. In addition, the enzyme's amino acid residues are responsible for forming the binding site that will lead to its specificity and selective properties, which will enable the enzyme to catalyze a reaction at a fantastic rate. Protein can also achieve their functionality by folding into compact and well-arranged structures. A protein can be found in the tertiary and quaternary structures, while, secondary structures only consist of α-helices, ß-sheets and turns. A simple combination of secondary structure elements was found in protein structure, called motif. These structures play a role in the different functions of proteins (Stiger et al., 1999).

Numerous studies have been reported on full length proteins with various functions inside living cells. A large protein normally consists of three-dimensional structure which is stable and well folded. A protein may also have several domains whereby a domain may exist in specific orientations to create different functions. With more than 35% of hydrophilic residues in a protein of more than 300 residues, the protein is likely to consist of more hydrophobic cores (George et al., 2005). This is an important characteristic for protein solubility. Generally, both natural and chemically synthesised large proteins are known to be active and functional. However, due to high demands of large protein in bulk or in relatively high concentration from the pharmaceutical and biosensor industry, there is an urgent requirement to fulfil this demand. Thus, to overcome this issue, a recombinant proteins have to be generated (Smyth et al., 2003). However, large-scale production of large protein can lead to high costs. In addition, producing recombinant proteins may sometimes lead to mismatches due to the extended length of the amino acid sequence in the original protein. Furthermore, the complex orientation that exists in the native protein may cause difficulty in recognising elements especially in the field of biosensors (Casset et al., 2003). Thus, developing a smaller protein that is related to a specific region of the native protein can be a good strategy for protein mimicking. Significant research efforts are devoted towards miniaturizing protein via truncations, corresponding to a particular specificity in purpose while simultaneously exploring their limited function.

Mini proteins, are low molecular weight proteins that active in specific biological processes. They have remarkable stability along with a high binding affinity and selectivity (Zoller et al., 2011). A better control on the mini protein could easily be activated with a lesser cross-reactivity in addition to being low cost and easier to generate. Small proteins also have fast turnaround time in production (Lipovsek et al., 2004).

Therefore, a small protein that mimics the active site of the enzyme can be developed without changing the specific characteristic of the native protein. In this research the targeted diamine oxidase (DAO) from *Athrobacter globiformis* had been miniaturized, for possible use as a biological receptor for specific biogenic amines in biocatalysis and biosensor technology.

Why diamine oxidase? In microorganism, diamine oxidases generally have a nutritional role in the utilization of primary amines as the sole source of nitrogen or carbon. They have been proposed to provide a signal in woung healing in plant (McGrath et al., 2009; Langley et al., 2006). While in mamals, they play an important role in regulating the intracellular level of amines such as neurotransmitter,detoxification, differentation, development of cell and some CuAOs are known to be tissue-specific (Duff et al., 2003; Matsumura et al., 2004; Lunelli et al., 2005). In facts, DAO was the enzyme originally identified to clear extrinsic histamine from crumble lung and liver (Murray et al., 1999). Furthermore, in application wise, the enzyme is used pharmacologically in many types of disease such as diabetes and Alzheimer's disease (Masini and Raimondi, 2009). However, the use of DAO as biosensor is becoming important nowadays. It concluded that the interest in DAO is increasing in several fields due to its involvement in numerous physiological and metabolic pathway that related to the biological function (Lunelli ey al., 2005). The versatility of DAO, provided a good reason why diamine oxidase was chosen to be the targeted protein. This knowledge is useful in understanding protein structure and function. It is hoped that this research may significantly contribute to the development of new biocatalysts in the future.

The aim of this research is to design a mini protein that functionally mimics the native enzyme. To achieve this objective, the following approaches have been undertaken.

1. To identify the selective active platform and scaffold of diamine oxidase.
2. To construct peptide structure *in silico*.
3. To experimentally validate the designed protein

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