CONSTRUCTION OF A STREP-TAG II MUTANT MALTOSE BINDING PROTEIN FOR REAGENTLESS FLUORESCENCE SENSING

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To my beloved parents, my family and my soulmate, my best friend; my husband and not forgetting to our newborn bub, Muhammad Rafiqi.

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ABSTRACT

Maltose binding protein (MBP) changes its conformational structure upon its ligand binding.This molecular recognition element that transduces a ligand-binding event into a physical one make MBP an ideal candidate for reagentless fluorescence sensing. MBP gene, (*malE)* was amplified from a pMaL-C4x plasmid vector and was fused to a Strep-Tag II pET-51b(+) vector. Strep-Tag II is a tag that will enable the MBP to be unidirectionally immobilized on solid supports. A cysteine mutant of the MBP was constructed by inverse PCR and the recombinant protein fusion was then purified by affinity purification using Strep-Tactin resin. To sense maltose binding, an environmentally sensitive fluorophore (IANBD amide) was covalently attached to the introduced thiol group. The tagged mutant MBP (D95C) was successfully generated and the protein was successfully purified with the expected molecular size of ~42 kDa observed on the SDS PAGE. The fluorescence measurements of the IANBD labeled of tagged mutant MBP (Strep-Tag II D95C) in the solution phase, showed an appreciable change in fluorescence intensity with dissociation constant, (K_d) of 7.6 ± 1.75 µM. Nonetheless, it could retain its ligand binding activity towards maltose. However, immobilization of Strep-Tag II D95C on solid surface suffered some limitation with the Strep-Tactin coated microwell plates because it did not give any dependable results to support the ligand binding activity of the site directed immobilized protein. Thus, this engineered mutant MBP (Strep-Tag II fused D95C) could be potentially developed for biosensor application with further improvement in protein immobilization method.

ABSTRAK

Protein pengikat maltosa (MBP) mengalami perubahan struktur konformasi semasa mengikat pada ligan. Molekul pengenalpastian yang menyebabkan transduksi pengikatan ligan kepada bentuk fizikal menjadikan MBP calon yang sesuai sebagai penderia pendaflour tanpa reagen. Gen MBP (*malE*) diamplifikasi daripada vektor plasmid pMaL-C4x dan kemudian digabungkan dengan Strep-Tag II yang terdapat pada vektor pET-51b(+). Strep-Tag II merupakan tag yang membolehkan MBP disekatgerak secara seragam kepada penyokong pepejal. Mutan MBP yang mempunyai satu residu sisteina telah dihasilkan melalui PCR berbalik dan gabungan protein rekombinan ini ditulen melalui penulenan afiniti yang menggunakan resin *Strep-Tactin*. Untuk mengesan pengikatan maltosa, flourofor yang sensitif pada persekitaran (IANBD amida) telah diikat secara kovalen kepada kumpulan tiol yang telah diperkenalkan pada protein itu. Mutan MBP yang bertag telah berjaya dijana dan melalui pemerhatian SDS-PAGE protein ini telah berjaya ditulen dengan saiz molekul ~42 kDa seperti yang dijangkakan. Ukuran pendaflour di dalam fasa larutan bagi mutan MBP yang bertag dan berlabel dengan IANBD menunjukkan perubahan ketara bagi keamatan pendaflour dengan pemalar penguraian K_d 7.6 ± 1.75 μM. Walau bagaimanapun, aktiviti pengikatan ligan terhadap maltosa boleh dikekalkan. Pemegunan protein Strep-Tag II D95C pada permukaan pepejal, berhadapan dengan beberapa kelemahan apabila piring mikrotelaga yang bersalut *Strep-Tactin* digunakan kerana ia tidak memberi keputusan yang dapat menyokong aktiviti pengikatan ligan oleh protein yang dipegunkan pada tapak khusus. Oleh itu, kejuruteraan mutan MBP gabungan Strep-Tag II-D95C berpotensi dibangunkan untuk aplikasi biopenderia dengan lebih penambahbaikan dalam kaedah pemegunan protein.

TABLE OF CONTENT

4 EXPRESSION AND PURIFICATION OF STREP-TAG II WILD TYPE MBP AND ITS MUTANT (D95C)

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ºC	-	Degree celcius
µg/ml	-	Microgram per millilitre
µl	-	Microliter
µM	-	Micromolar
bp	-	Basepair
BLAST	-	Basic local alignment search tool
$CaCl_2$	-	Calcium chloride
cm	-	centimeter
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleotides
dsDNA	-	double stranded Deoxyribonucleic acid
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetraacetic acid
ELISA	-	Enzyme-linked immunosorbent assay
g	-	gram
HRP	-	Hydrogen peroxidase
IANBD	-	N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4 -yl)ethylenediamine
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
K_2HPO_4	-	Dipotassium hydrogen phosphate
KCl	-	Potassium chloride
K_d	-	Dissoication constant
kDa	-	kilodalton
KH_2PO_4	-	Potassium dihydrogen phosphate
KOAc	-	Potassium acetate

LB	-	Luria Bertani
LBA	-	Luria Bertani Agar
M	-	Molar
$MgSO_4.7H_2O$	-	Magnesium sulfate heptahydrate
mM	-	Milimolar
$MnCl_2$	-	Manganese chloride
MOPS	-	3-(N-morpholino)propanesulfonic acid
NaCl	-	Sodium chloride
nm	-	Nanometer
OD	-	Optical density
PAGE	-	Polyacrylamide Gel Electrophoresis
PBS	-	Phosphate buffer saline
PCR	-	Polymerase chain reaction
PMSF	-	Phenylmethanesulfonyl fluoride
RbCl	-	Rubidium chloride
rpm	–	Rotation per minute
SDS	-	Sodium dodecyl sulfate
TAE	-	Tris base, acetic acid, EDTA buffer
UV	-	Ultraviolet
V	-	Voltage
v/v	-	Volume per volume
w/v	-	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Introduction

Biosensor is an analytical device that functions by coupling a biological sensing element with a detector system using a transducer (Chauhan *et al.*, 2004). It involves exploiting the recognition and detection system of a biology component for a target molecule or macromolecule with a transducer that converts the biological recognition event into an output signal (Collings *et al.*, 1997). The signal can be electrical, optical or thermal and is converted by a suitable transducer into a measurable electrical parameter such as electrical or current. Since its establishment, biosensors have been widely used in diagnostics, pharmaceutical research, agriculture, food safety, environment and industrial monitoring (Luong *et al.*, 2008).

There have been some limitations in producing biosensors because each device is unique and requires an amount of time and optimization. This is because developments of most biosensors involve the identification of natural specificity of a biological component for an analyte and the discovery of a suitable signal transducer that is adapted to the macromolecule (Chauhan *et al.*, 2004). Hence, protein engineering techniques are being used to overcome this issue where signal transduction properties of biological molecules are being modified to adapt the detector instrumentation rather than adapting the detector instruments to the unique

requirements of each natural molecule. This is achieved by integrating a functional group that gives simple signal-transduction mechanism such as optical or electrical, to the protein itself (Hellinga and Marvin, 1998).

Development of sensor devices requires incorporation of sensing proteins into a detector element by encapsulation or surface immobilization on a suitable material for interfacing with detectors (De Lorimier *et al.*, 2006). It is crucial for the protein to be immobilized for reagentless biosensing and for further application in biosensor such as protein microarray. Thus, with protein engineering techniques, enzymes and proteins can be chemically modified to make them more stable and more specific with more regular interface for immobilization. A protein may be modified so that its active site points outwards from the sensor surface once the protein is immobilized because the active sites need to be accessible to the analyte (Collings *et al.*, 1997). Thus, in this research, rational protein engineering techniques will be applied to molecular engineer proteins for unidirectional immobilization on functionalized surfaces and to investigate its protein activity. Moreover, it will assess the applicability of immobilized protein for biosensing application for high throughput analysis of analyte.

The protein used in this research is maltose binding protein (MBP) which is in the superfamily of periplasmic binding protein (PBP) that is extensively studied as receptors for sensor applications (Hellinga *et al.*, 1998). This is because PBP superfamily has a remarkable adaptablity for their cognate ligands and can be designed to bind nonnatural ligands (Marvin and Hellinga, 2001). The structure of PBPs both with and without their ligands bound, has been described as a 'Venus Flytrap' where the two lobes of the protein will close upon the ligand, entrapping it (Gilardi *et al.*, 1994). This molecular recognition element that transduces a ligand-binding event into a physical event makes it suited for biosensor applications such as reageantless fluorescent biosensing. MBP will be altered genetically to construct a regeantless biosensor so that a reporter group (fluorophore) may be covalently linked to MBP. The fluorophore will respond to the ligand binding event of the labeled protein and this response is measured by fluorescence intensity changes.

It is worth noting that, to the extent of our knowledge, all of the reported works describing signal transduction by fluorophore labeled PBPs have been studied without their linkage to solid supports (Brune *et al.*, 1994; Gilardi *et al.*, 1994; Marvin *et al.*, 1997; Hellinga and Marvin, 1998; Marvin and Hellinga, 2001). In this research, protein immobilization will be done by exploiting the affinity tag by genetic engineering. Affinity tags are widely used in biotechnology to assist purification of recombinant protein. The MBP will be fused to an affinity tag, Strep-Tag II to aid in purification and site-directed immobilization of the MBP onto functionalized solid surfaces. Strep-Tag II consists of eight amino acids fusion tag that will bind to Strep-Tactin protein (Merck Biosciences, 2007). The Strep-Tag II will act as a handle that will enable the attachment of MBP to a Strep-Tactin surface on microwell-plates for optimal ligand binding.

1.2 Research objectives

The ultimate aim of this work was to construct tagged fusion proteins for site directed immobilization onto surfaces with a view to investigate their potential for biosensor applications. To achieve this, the objectives were as follows:

1. To mutate the Maltose Binding Protein (MBP) for the development of a reagentless fluorescence sensing system for maltose.

2. To overexpress and purify Strep-Tagged II fusion MBP and its mutant via affinity chromatography

3. To determine the ligand binding activity of fluorophore-labeled mutant MBP via fluorescence intensity measurements.

1.3 Significance of Research

The immobilization of proteins onto solid surfaces remains a critical aspect in the development of biosensors. Furthermore, to the extent of our knowledge, all of the reported works describing signal transduction by fluorophore labeled PBPs have been studied without their linkage to solid supports. Thus, in this research, MBP was first mutated to construct a reagentless biosensor for maltose sensing. The Strep-Tag II fused to the mutated MBP enabled the attachment of the ligand binding protein onto a functionalized solid surface. The ability of the immobilized protein to sense ligand binding was subsequently assessed.

1.4 Scope of Research

To fulfill the objectives of this research, there were four main experimental steps in laboratory work that needed to be done. Firstly, the construction of plasmids containing wild type *malE* gene and its variant were done. This involved amplification of *malE* gene, site-directed mutagenesis of *malE* gene and cloning. The next step was expression and purification of wild type MBP and its mutant; which involved optimizing protein expression to produce the optimal amount of protein and purification of the protein by affinity purification. The third step was to analyze the ligand binding characteristics of wild type MBP and its mutant via fluorescence. Fluorescence measurements involved labeling the mutant MBP with fluorophore and analyzing the ligand binding characteristic of MBP via fluorescence. The final step was to analyze the ligand binding characteristics of the immobilized protein via fluorescence. In this final step, the labeled mutant protein was immobilized onto a functionalized microwell plate and its ability to sense maltose via fluorescence intensity changes was determined.

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