SILVER INHIBITION ON RECOMBINANT FLAVIN REDUCTASE FROM *Citrobacter freundii* A1

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To my beloved mother and father,
Siti Hajar Juhar and Mamat Ngadirin,
My siblings,
My lovely friends,
And
My supportive supervisor,
Dr Chan Giek Far

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Thank to Allah, due to His blessing, finally I able completing my thesis with the time given. Besides that, thank you to my beloved parents who always support me by giving advices and financial support from the beginning till the end. To my supportive supervisor, Dr. Chan Giek Far, thanks a lot because willing to help and teach me to complete my project. Her guidance and point of view led me to complete this project successfully.

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I hope, this thesis entitled ‘Silver Inhibition on Recombinant Flavin Reductase from *Citrobacter freundii* A1’ can give beneficial knowledge and understanding for other students who are interested with this challenging research.

ABSTRAK

Dalam kajian ini, flavin reductase (Fre) yang berasal dari *Citrobatter freundii* A1 telah diekspreskan dalam perumah *E.coli* DH5α dengan 1mM IPTG. Protein rekombinan ini telah digabungkan dengan 6 Histidine-tag proteinyang memudahkan penulenan enzim tersebut dengan menggunakan IMAC. Flavin reductase memangkinkan penurunan flavin bebas dengan menggunakan NADH untuk menghasilkan flavin bebas yang terturun. Penulenan rekombinan favin reductase dilihat menggunakan 15 % SDS-PAGE dan berat molekul protein sasaran yang telah ditulenkan adalah kira-kira 27.04 kDa. Dalam kajian ini, aktiviti flavin reductase dalam pengoksidaan NADH telah diukur pada 340 nm ($\varepsilon 340 = 6.22\ mM^{-1}\ cm^{-1}$) . Kesan ion perak dari perak nitrat ($AgNO_3$) ke atas aktiviti flain reductase telah dikaji. Apabila kepekatan ion perak meningkat, aktiviti relatif dan kelajuan flavin reductase dalam pengoksidaan NADH semakin berkurang. Ion perak berkemungkinan merencat aktiviti flavin reductase dengan mekanisma tidak berbalik melalui ikatan yang tidak spesifik terhadap asid amino yang berbeza. Kesimpulannya, ion perak boleh menjadi perencat yang berpotensi terhadap flavin reductase dari *C. freundii* A1.

ABSTRACT

In this study, the flavin reductase (Fre) of *Citrobatter freundii* A1 was expressed in *E. coli* DH5α host with 1 mM IPTG induction. This recombinant protein was fused to 6xHis-tag, thus promising easier IMAC purification of the expressed enzyme. Flavin reductase catalyzes the reduction of free flavin using NADH to produce free reduced flavin. The purity of recombinant flavin reductase was observed using 15 % SDS-PAGE and the molecular weight of the target protein was determined to be 27.04 kDa. In this study, flavin reductase activity was measured at 340 nm (ε_{340} = 6.22 $mM^{-1}cm^{-1}$) due to the oxidation of NADH. The effect of silver ions from silver nitrate ($AgNO_3$) on the activity of flavin reductase was determined. As the concentration of silver ions increased, the relative activity and velocity of flavin reductase reaction decreased over time. Silver ions may inhibit flavin reductase irreversibly via unspecific binding to different amino acids. In conclusion, silver ions could be a potent inhibitor of flavin reductase of *C. freundii* A1.

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LIST OF SYMBOLS AND ABBREVIATIONS

Ag^+	Silver ion
$AgNO_3$	Silver nitrate
ATG	Start codon
AR27	Acid red 27
ClHgBzOH	*p*-chloromercuri-benzoate
CHO	Chinese Hamster Ovary
CV	Column volume
Cu^{2+}	Cuprum ion
Cys	Cystein
DBT	Dibenzothiophene
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
ETC	Electron transport chain
Fre	Flavin reductase
FMN	Flavin mononucleotide
FAD	Flavin adenine dinucleotide
FNR	ferredoxin–$NADP^+$ reductase
Fe (II) / (III)	Ferum (II) / (III)
HIC	Hydrophobic Interaction Chromatography
His	Histidine
H_2O_2	Hydrogen peroxide
HEK	Human Endothelial Kidney
IPTG	Isopropyl-B-D-thiogalactopyranoside
IMAC	Immobilize metal ion affinity chromatography

IEC	Ion Exchange Chromatography
IEF	Isoelectric Focusing
kDa	kilo Dalton
LBA	Luria betani + ampicillin
MalNEt	*N*-ethylmaleimide
MCS	Multiple cloning site
M / mM	Molar / milimolar
NAD(P)H	Reduced pyridine nucleotide
Ni-NTA	Nickel-nitrilotriacetate
Ni^2	Nickel ion
OD	Optical density
ox	oxidized
PCD	Programmed cell death
P_i	Phosphate
ROS	Reactive oxygen species
red	reduced
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	Size Exclusion Chromatography
Ser	Serine
UQ	Ubiquinone
%	Percentage
µg/mL	Microgram/milliliter
˚C	Degree Celsius
g	Gram
mg/mL	Milligram/milliliter
µL	Micro liter
nmol	Nanomole
nm	Nanometer
U/mg	Specific activity of enzyme

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CHAPTER 1

INTRODUCTION

1.1 Background study

Flavin reductase (Fre) is a part of multienzyme system that reduces the Fe (III) center of ribonucleotide reductase to Fe (II) (Giannis *et al.,* 1991). Flavin reductase is also known as NADPH:flavin oxidoreductase. The enzyme is an oxidoreductase that catalyzes the redox reaction in which oxygen or hydrogen atoms are gained or lost. In physiological functions, flavin reductase is directly associated with the released product which is the reduced free flavin (Fontecave *et al.,* 1994).

Other than that, this enzyme is also known as riboflavin mononucleotide reductase, flavin mononucleotide reductase, NADPH dehydrogenase (flavin), NADPH: riboflavin oxidoreductase, NADPH-dependent FMN reductase, and NADPH-flavin reductase (Yubisui *et al.,* 1987).

In general, Fre catalyzes the reduction of free flavin (i.e riboflavin, FMN, FAD) by using reduced pyridine nucleotides such as NADPH or NADH to produce reduced flavin. Fieschi *et al.* (1995) described the variety of potential uses of free

flavins in nature as an electron transfer mediator. During iron metabolism, bioluminescence, oxygen activation, and activation of ribonucleotide reductase, reduced free flavin was used extensively to maintain the biological metabolism. Figure 1.1 describes the reduction mechanism catalyzed by flavin reductase.

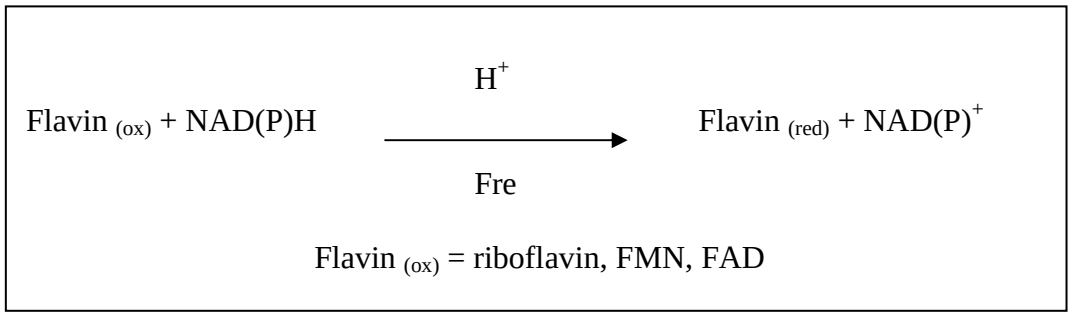

Figure 1.1 Flavin reductase catalyzes the reduction of riboflavin in the presence of reduced pyridine nucleotide (NADPH) to produce free reduced flavin (Adapted from Fieschi *et al.,* 1995).

In addition, this valuable enzyme was found to be involved in the desulfurization process of fossil fuels (Gray *et al.*, 1996) and antibiotic biosynthesis (Parry and Li 1997). Moreover, flavin reductase activity was also found in various human tissues such as liver, heart, kidney, and lung. The activity was also detected in erythrocytes of human (Quandt and Hultquist, 1994).

Since there are numerous functions of flavin reductase either *in vivo* or *in vitro*, the inhibitory effects towards this enzymes activity need to be considered. The activity of flavin reductase could be inhibited by sulfhydryl reagents such as *N*-ethylmaleimide (MalNEt), iodoacetic acid, and *p*-chloromercuri-benzoate (ClHgBzOH). These findings suggested that cysteines (Cys) are important residues of the active site of the enzyme (Mazhul and Danilov, 1994; Fontecave *et al.*, 1987; Jablonski and DeLuca, 1978). Flavin binding site has thiol groups which is the best candidate to be inhibited by silver ions.

Matsumura *et al.*, (2003) studied about the bactericidal action of silver zeolite and silver nitrate. In their findings, both silver zeolite and silver nitrate showed inhibitory effects on bacterial survival. Silver ion is a type of heavy metal that causes deposition of proteins *in vitro* and *in vivo*. Entrance of silver ions into the cell could cause cell damage, thus leading to death. Through the observation by electron microscopy and X-ray microanalysis, detachment of cytoplasm membrane from cell wall, condensation of DNA, and deposition of proteins inside the cell occurred during silver inhibition (Feng *et al.*, 2000).

The exact mode of Ag^{+} bactericidal action is still unknown eventhough the inhibitory effect was reported. These inhibitors react with thiol group of the enzymes such as NADH dehydrogenase II or interact with DNA binding resulting in pyrimidine dimerization. Both of these reactions could cause an apoptosis by the production of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) intracellularly (Cabiscol *et al.*, 2000).

1.2 Problem statement

Recombinant proteins act as important and crucial tools in studying the biological process *in vitro*. Since there are many functions of Fre in nature, (i.e luminescence reaction) the purified recombinant flavins reductase will enable us to explore the enzyme from various aspects. Obtaining desired protein with high yield and purity becomes challenging aspect nowadays. Therefore, expression system like bacteria was extensively used to produce Fre recombinant protein.

Previous observation suggested that cytoplasm membrane is the main site for biocidal activity of Ag^{+}. In the cytoplasm membrane there are lots of embedded proteins associated with respiration and proton motive force. Therefore, this study

was carried out to investigate the effect of silver ions on flavin reductase which is a cytoplasmic enzyme that catalyzes the reduction of free flavin (Tortora *et al.*, 2007, Trudy and James 2003). In addition, effect of silver ions on flavin reductase activity remains unknown. Previous study had focused on bactericidal effect of silver ions on bacterial growth and until now there is no study about silver inhibitions on flavin reductase. Based on former findings, it was suggested that flavin binding site that contains thiol groups may probably be the best site to be attacked by silver ions. However, in the recent year, Tamer *et al.* (2002) had discovered that silver ions have different binding affinities with other 20 amino acids instead of cysteine itself.

Flavin reductase (*fre*) gene from *Citrobacter freundii* A1 was formerly cloned in pQE-82L vector and successfully transformed into *E. coli* DH5α host (Narges 2010). pQE-82L is a 4752-bp expression vector and has fusion protein, 6xHis-tag located between the start codon, ATG and multiple cloning sites (MCS). The availability of histidine (His) in the vector together with Fre target protein could give an opportunity to purify the recombinant protein using immobilized metal ion affinity chromatography (IMAC). Hence, this allows the flavin reductase assay as well as the silver inhibition study to be carried out on the purified enzyme.

1.3 Objectives

The objectives of this study are:

1) To express the recombinant flavin reductase in *E. coli* DH5α containing pQE-82L expression vector
2) To purify the expressed recombinant flavin reductase
3) To study the effect of silver nitrate ($AgNO_3$) on recombinant flavin reductase activity

1.4 Scope of works

Flavin reductase from *Citrobacter freundii* A1 was expressed in *E. coli* DH5α. Upon sub-culturing, the expression of recombinant flavin reductase was induced with Isopropyl-B-D-thiogalactopyranoside (IPTG). Since 6xHis-tagged protein has affinity towards transition metals, immobilized metal ion affinity chromatography (IMAC) was performed to purify this recombinant protein, and the purity was further analyzed with SDS-PAGE. In order to know the activity of the desired protein, enzymatic study was performed and the absorbance was detected using UV-Visible Spectrophotometer (Shimadzu) at 340 nm. The effect of silver ions on the recombinant flavin reductase was determined using the purified enzyme.

1.5 Significance of study

This is the first attempt to express flavin reductase from *C. freundii* A1 in *E. coli* DH5α. This study is carried out to express and purify Fre recombinant protein. As most microorganisms possess the enzyme, silver inhibition study on flavin reductase will contribute to the understanding of possible bactericidal mechanism of this antibacterial agent. Therefore, throughout this study, it will enable us to understand the effect of silver ions on purified recombinant flavin reductase activity.

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