UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION AND CHARACTERIZATION OF FERREDOXIN NADP+ REDUCTASE FROM *BACILLUS sp.*

SARAH SYAMIMI OTHMAN

FBSB 2015 75

IDENTIFICATION AND CHARACTERIZATION OF FERREDOXIN NADP^{+} REDUCTASE FROM *BACILLUS sp.*

SARAH SYAMIMI BT OTHMAN

163043

DEPARTMENT OF BIOCHEMISTRY

FACULTY OF BIOTECHNOLOGY AND BIOMOLECULAR SCIENCES

UNIVERSITY PUTRA MALAYSIA

2015

PENGESAHAN

Dengan ini adalah disahkan bahawa laporan bertajuk "Identification and Characterization of Ferredoxin $NADP^+$ Reductase from *Bacillus sp*." telah disiapkan serta dihantar kepada Jabatan Biokimia oleh Sarah Syamimi Bt Othman sebagai syarat untuk kursus BCH 4999 Projek dari Fakulti Bioteknologi dan Sains Biomolekul, Universiti Putra Malaysia.

Disahkan oleh:

…… Tarikh:……..

Prof. Dato' Dr Abu Bakar Salleh

Penyelia Projek

…….. Tarikh:……

Prof. Dato' Dr Abu Bakar Salleh

Ketua

Jabatan Biokimia

Fakulti Bioteknologi dan Sains Biomolekul

Universiti Putra Malaysia.

ACKNOWLEDGEMENT

I would love to express my heartfelt gratitude to all those people who have been accompanying me in this final year project and have been assisting me and making it an unforgettable experience. My deep sense of appreciation to Prof. Dato' Dr Abu Bakar Salleh, Head of Department Biochemistry, for his counsel and helpful supervision during the project. I would like to express my sincere thanks and gratefulness to my mentor Miss Ang Swi See, for her guidance and constant supervision as well as for providing necessary information regarding the project. I am heartily thankful to Mr Hisham, Lab Manager, for the encouragement and his kind co-operation during my project. I also would like to express my warm thanks to Kak Ain who always accompanied me during night and weekends in laboratory. I would also like to thank all the partner lab members, Fatin Syuhadah, Nurul Izzati, Nalini, Afron, Kak Nida, Kak Yati, Kak Chik, Kak Ema, Kak Zurith, Abang Anas, Abang Adi and Kak Ashwini who were always there at the need of the hour and provided me all the help and facilities, which I required for this work and for providing help in my laboratory work. I also extend my deepest thanks to my family especially my mother, Rosinah Bt Muhamad and my father, Othman B Taip because without them I cannot be here.

TABLE CONTENTS

LIST OF TABLES

PAGES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	Base pair
°C	Degree Celsius
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
g	Gram
IPTG	Isopropyl-B-thioga-lactopyranoside
kDa	kiloDalton
L	Liter
LB	Luria-Bertani
mg	Milligram
µg	Microgram
ml	Milliliter
M	Molar
mM	Milli Molar
µl	Microliter
nm	Nanometer
OD	Optical density
PAGE	Poly-acrylamide gel electrophoresis
%	Percentage
Rpm	Rotation per minute
g	Relative centrifugal force in unit of x*g*
SDS	Sodium dodecyl sulphate
TEMED	N,N,N,N' tetramethyl-ethylene diamine

ABSTRACT

The aim of this experiment was to identify and characterize the enzyme ferredoxin $NADP^+$ reductase from *Bacillus sp*. The gene was cloned and expressed in *Escherichia coli* system by using pEt102 TOPO vector as a host. The recombinant *E. coli* was cultured in Luria Bertani broth, and it was being induced with isopropyl-β-thioga-lactopyranoside (IPTG). Next, the expression culture was spun down by high speed centrifugation. Pellet was collected and resuspended with binding buffer and then sonicated. Centrifugation was repeated until the supernatant was collected and loaded on nickel-Sepharose chromatography. The purified ferredoxin $NADP^+$ reductase was a protein with molecular mass of approximately 54kDa based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). NADPH was a good electron donor meanwhile the electron acceptor was a 2,6-Dichlorophenolindophenol (DCPIP) in a diapharose assay. Furthermore, the maximum activity for this enzyme was observed at 25℃ and at pH 7. It had been proven that this enzyme could withstand for 120 minutes at 25℃ and at pH10. Results from experiment suggested that ferredoxin $NADP^+$ reductase can be grouped as an oxidoreductase enzyme which had a capability to facilitate the reaction between $NADP^+$/ NADPH with ferredoxin.

ABSTRAK

Tujuan eksperimen ini adalah untuk mengenal pasti dan mencirikan enzim feredoksin $NADP^+$ reduktase yang berasal daripada *Bacillus sp*. Gen ini telah diklonkan dan diexpresskan dalam sistem *Escherichia coli* sebagai perumah dengan menggunakan vektor pEt102 TOPO. Selepas itu, rekombinan *E. coli* telah ditumbuhkan di dalam media Luria Bertani, dan ia telah dirangsangkan dengan menggunakan Isopropil-β-thioga-lactopyranoside (IPTG). Seterusnya, sell hidup telah diemparkan dengan menggunakan pengempar berkelajuan tinggi. Pelet dikumpulkan dan diampaikan dengan menggunakan penimbal pengikat dan kemudian disonikasikan. Pengemparan ini diulang sehingga supernatan dikumpulkan dan dimuatkan pada kromatografi nikel sepharose. Dalam kajian ini, feredoksin $NADP^+$ reduktase merupakan protein dengan kandungan jisim molekul kira-kira 54 kDa dengan menggunakan elektroforesis gel poliakrilamida-sodium dodesil sulfate (SDS-PAGE). NADPH merupakan penderma elektron yang bagus sementara penerima elektron adalah 2,6-Diklorofenolindofenol (DCPIP) dalam cerakin diafharosa. Tambahan pula, aktiviti maksimum bagi enzim ini diperhatikan pada suhu 25 ℃ dan pada pH 7. Ia telah dibuktikan bahawa enzim ini boleh bertahan sehingga 120 minit pada 25 ℃ dan pH 10. Keputusan experimen menunjukkan bahawa feredoksin $NADP^+$ reduktase boleh dikelaskan sebagai enzim oksidoreduktase yang mempunyai keupayaan untuk memudahkan tindak balas antara $NADP^+$ / NADPH dengan feredoksin.

CHAPTER 1

1.0 INTRODUCTION

1.1 GENERAL

Ferredoxin $NADP^+$ reductase (FNR) is an enzyme that catalyzes the chemical reaction between ferredoxin and NADPH. At the end of the reaction it will form a product called oxidized ferredoxin and NADPH. This enzyme contains FAD as a flavin cofactor to increase the rate of reaction. Below is equation for reaction: (Cassan, *et. al*., 2005; Berg, *et. al*., 2007)

$$2 \text{ ferredoxin (red)} + NADP^+ + H^+ \leftrightarrow 2 \text{ ferredoxin (ox)} + NADPH$$

This enzyme belongs into a group of oxidoreductases. An electron donor for this enzyme is iron-sulfur proteins while the electron acceptors are $NADP^+$ or NAD^+. Furthermore, ferredoxin $NADP^+$ reductases can be classed into two unrelated protein families. The first protein family is plant type ferredoxin $NADP^+$ reductase which also can be further divided into two subclasses which are plastidic FNRs in plants and the bacterial FNRs. The second protein family is glutathione-reductase-type FNRs which usually found in the mitochondria of eukaryotes (Aliverti, *et. al*., 2008).

Commonly, this enzyme has several roles and one of its functions is the enzyme takes part in photosynthesis in order to transport the electrons between flavodoxin or ferredoxin and NADPH (Knaff and Hirasawa, 1991; Sétif, 2001). Another function of this enzyme can be seen in non-photosynthetic organisms where the FNR provides reduced ferredoxin for various metabolic pathways including nitrogen fixation, terpenoid biosynthesis, steroid metabolism, oxidative stress response, and iron–sulfur protein biogenesis (Aliverti, *et. al*., 2008).

There are a few disadvantages regarding of ferredoxin and ferredoxin $NADP^+$ reductase in the reaction. For instance, in the electron transport chain, the rate limiting step is the release of the first oxidized ferredoxin molecule after the FAD is being reduced with one electron. This step can be inhibited by the presence of oxidized ferredoxin and stimulated by the existence of $NADP^+$ (Carrillo, *et. al*., 2003). Next, the binding affinity of the enzyme towards ferredoxin can be decreased by binding of $NADP^+$ to the enzyme (Medina, 2009). Furthermore, in the absence of light, ferredoxin $NADP^+$ reductase is inhibited thus electron cycling from ferredoxin to NADPH cannot occur (Talts, *et. al*., 2007). In addition, the second-order rate constant for the first reduction of ferredoxin-$NADP^+$ reductase was 20% slower when $NADP^+$ is present, in line with the existence of ternary complexes (ferredoxin-$NADP^+$ reductase)-$NADP^+$-ferredoxin.

Spectrophotometric assay which is known as a continuous assay can be used for detection in enzyme assay. The procedure is simple, selective, non-destructive and also because it is very sensitive. By using spectrophotometer it can be used to measure the concentration of NADH changes when it react with ferredoxin at 340nm (Stryer, 1988).

In addition, one of a successful biotechnological application of enzyme reductase is ferredoxin $NADP^+$ reductase can be a drug to fight against apicomplexia human parasites. This parasite eventually has the ability to spread diseases such as malaria and congenital disease. This enzyme will reside inside the apicoplast which is an organelle that being required for the survival of the pathogen (Aliverti, *et. al*., 2008). One of the factors why ferredoxin $NADP^+$ reductase can be used as potential drug is because in host organism it does not have any homologous proteins. Moreover, apicomplexan and plant enzymes have mostly identical structure thus if the electron

flow from ferredoxin $NADP^+$ reductase to ferredoxin is being blocked it will eventually may kill the parasites itself. The electron flow from ferredoxin $NADP^+$ reductase to ferredoxin is being blocked by either inhibit the ferredoxin $NADP^+$ reductase activity or it interaction between the substrate itself which is ferrodoxin (Seeber, *et al*., 2005).

1.2 PROBLEM STATEMENT

The characteristic and function of ferredoxin $NADP^+$ reductase from *Bacillus sp.* are still unknown.

1.3 OBJECTIVE

1. To purify and characterize ferredoxin $NADP^+$ reductase from *Bacillus sp.*

BIBLIOGRAPHY

Aliverti, A., Jansen, T., Zanetti, G., Ronchi S., Herrmann, R.G., Curti, B. (1990). Expression in *Escherichia coli* of ferredoxin : $NADP^+$ reductase from spinach. Eur. J. Biochem. 191, 551 -555.

Aliverti, A., Pandini, V., Pennati, A., Matteo, D. R., Zanetti, G. (2008). Structural and functional diversity of ferredoxin-$NADP^+$ reductases. Arch. Biochem. Biophys. 474, 283-291.

Berg, Jeremy M., Tymoczko, John L., Stryer, Lubert. (2007). Biochemistry, 6th ed., New York: W.H. Freeman.

Brunings, A. M., Gabriel, D. W. (2003). *Xanthomonas citri*: breaking the surface. Mol Plant Pathol. 4, 141-157.

Cassan, N., Lagoutte, B., Sétif, P. (2005). Enzyme catalysis and regulation: kinetics of electron transfer, transient intermediates, and catalytic activities studied by flash-absorption spectroscopy with isolated photosystem I and ferredoxin. J. Biol. Chem. 280, 25960-25972.

Carrillo, N. and Ceccarelli, EA. (2003). Open questions in ferredoxin-$NADP^+$ reductase catalytic mechanism. Eur J Biochem 270 (9), 1900–15.

Ghosh, A., Bhardwaj, M., Satyanarayana, T., Khurana, M., Mayilraj, S., Jain, R.K. (2007). *Bacillus lehensis sp*. nov., an alkalitolerant bacterium isolated from soil. Int J Syst Evol Microbiol. 57 (2), 238-42.

Graham, J. H., Gottwald, T. R., Cubero, J., Achor, D. S. (2004). *Xanthomonas axonopodis* pv. *citri*: factors affecting successful eradication of citrus canker. Mol Plant Pathol 5, 1-15.

Ikeda, T., Nakamura, M., Arai, H., Ishii, M., Igarashi, Y. (2009). Ferredoxin-$NADP^+$ reductase from the thermophilic hydrogen-oxidizing bacterium, *Hydrogenobacter thermophiles* TK-6. FEMS Microbial Lett. 297, 124-130.

Knaff, D.B., Hirasawa, M. (1991). Ferredoxin-dependent chloroplast enzymes. Biochim. Biophys. Acta 1056, 93-125.

Komori, H., Seo, D., Sakurai, T., Higuchi, Y. (2010). Crystal structure analysis of *Bacillus subtilis* ferredoxin-$NADP^+$ oxidoreductase and the structural basis for its substrate selectivity. (2010). Protein Science. 19, 2279-2290.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Medina, M. (2009). Structural and mechanistic aspects of flavoproteins: photosynthetic electron transfer from photosystem I to $NADP^+$. FEBS J 276 (15), 3942–58.

Pandini, V., Caprini, G., Thomsen, N., Aliverti, A., Seeber, F., Zanetti, G. (2002). Ferredoxin-$NADP^+$ reductase and ferredoxin of the protozoan parasite *Toxoplasma gondii* interact productively in vitro and in vivo. J. Biol. Chem. 277, 48463–48471.

Park, Y. J., Yoo, C. B., Choi, S. Y., Lee, H. B. (2006). Purifications and characterizations of a ferredoxin and its related 2-Oxoacid: Ferredoxin Oxidoreductase from the Hyperthermophilic Archeon, *Sulfolobus solfataricus* P1. Journal of Biochemistry and Molecular Biology. 39, 1, 46-54.

Savage, N. (1957). The Preparation and Properties of a Soluble Disphosphopyridine Nucleotide Cytochrome C Reductase. Biochem. J. 67, 146-155.

Seo, D., Kamino, K., Inoue K., Sakurai H. (2004). Purification and characterization of ferredoxin-$NADP^+$ reductase encoded by *Bacillus subtilis* yumC. Arch Microbiol. 182(1), 80-89.

Seo, D., Asano, T., Komori, H., Sakurai, T. (2014). Role of the C-terminal extension stacked on the re-face of the isoalloazine ring moiety of the flavin adenine dinucleotide prosthetic group in ferredoxin-$NADP^+$ oxidoreductase from *Bacillus subtilis*. Plant Physiology and Biochemistry. 1-6.

Seeber, F., Aliverti, A., Zanetti, G. (2005). The plant-type ferredoxin-NADP$^+$ reductase/ferredoxin redox system as a possible drug target against apicomplexan human parasites. Curr Pharm Des 11 (24), 3159–72.

Sétif, P. (2001). Ferredoxin and flavodoxin reduction by photosystem I. Biochim. Biophys. Acta 1507, 161-179.

Stryer, L. (1988). Biochemistry, 3rd ed., chapter 8. W.H. Freeman and Co. SF.

Talts, E., Oja, V., Rämma, H., Rasulov, B., Anijalg, A., Laisk, A. (2007). Dark inactivation of ferredoxin-NADP$^+$ reductase and cyclic electron flow under far-red light in sunflower leaves. Photosynth Res 94 (1), 109–20.

Tondo, M. L., Musumeci, M. A., Delprato, M.L., Ceccarelli, E. A., Orellano, E. G. (2011). Structural-functional characterization and physiological significance of ferredoxin-NADP$^+$ reductase from *Xanthomonas axonopodis* pv. *citri.* PLoS ONE 6(11), e27124. doi:10.1371/journal.pone.0027124.

Wojtaszek, P. (1997). Oxidative burst: an carly plant response to pathogen infection. Biochem J. 322, 681-692.

Yan, Z., Nam, Y. W., Fushinobu, S., Wakagi, T. (2014). *Sulfolobus tokodaii* ST2133 is characterized as a thioredoxin reductase-like ferredoxin:NADP$^+$ oxidoreductase. Extremophiles 18, 99–110.

Yeom, J., Jeon, C. O., Eugene L., Madsen, Woojun. (2009). Ferredoxin-$NADP^+$ reductase from *Pseudomonas putida* functions as a ferric reductase. J. Bacteriol. 191(5), 1472-1479.

Yoch, D. C. (1973). Purification and characterization of ferredoxin-nicotinamide adenine dinucleotide phosphate reductase from a nitrogen-fixing bacterium. J. Bacteriol. 116(1), 384-391.