GENE EXPRESSION, CHARACTERIZATION AND *IN SILICO* STUDIES OF DELTA FATTY ACID DESATURASES

By

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Chair : Professor Raja Noor Zaliha Raja Abd. Rahman,D.Eng.
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Fatty acid desaturases (FAD) catalyze desaturation reactions by the insertion of double bonds into the fatty acyl chain, producing unsaturated fatty acids. Though soluble FAD has been studied widely in higher living organisms, there are very limited studies of membrane FAD due to the difficulty of generating recombinant desaturases as well as their higher tendency to become toxic cells. Membrane FAD is predominant, as they play a crucial role in the synthesis of polyunsaturated fatty acids and offer nutritional benefits to humans. In this study, a range of fatty acid desaturases consisting of Δ6, Δ12, and Δ15 FAD was chosen from different organisms which exists abundantly to pave way for characterization. Δ6 FAD was isolated, and PCR amplified from *Anoxybacillus geothermalis*, cloned in pGEX-4T1 vector and expressed in *E. coli* BL21 (DE3) with the expected amplicon of 1104 bp encoding for 368 amino acids obtained with the restriction enzymes *Bam*HI and *Eco*RI. The 1230 bp open reading frame of Δ12 FAD coding for 410 amino acid sequences from *Brassica napus,* had been successfully synthesized. It was cloned in pET-51b(+) with restriction enzymes *Sal*I and *Not*I and expressed in *E. coli* BL21 (DE3) at 37 °C in intracellular and inclusion body fractions when induced with 0.5 mM IPTG, with an expected size of 49 kDa. Recombinant Δ12 FAD was successfully purified in two steps of chromatography: hydrophobic interaction chromatography (HIC) and ion exchange chromatography (IEX) with resin Butyl-S Sepharose 6 Fast Flow and SP Sepharose Fast Flow, respectively with a total protein yield of 1.55 mg/mL. The characterization of recombinant Δ12 FAD revealed desaturase activity of Δ12 FAD could produce linoleic acid from oleic acid at a retention time of 17.6 with a composition of 47%. Analysis of circular dichroism (CD) showed Δ12 FAD was made up of 47.3% and 0.9% of α-helix and β-sheet secondary structures, respectively. The predicted Tm value was 50.2 °C. Δ15 FAD had been synthesized from

Synechocystis sp., a cyanobacterium and cloned in pET-51b(+) with restriction enzyme *Sal*I and *Not*I with expected size around 1155 bp coding for 385 amino acid sequences. The protein was expressed in intracellular and inclusion body fractions in *E. coli* BL21 (DE3) at 30 °C when it was induced by 1.0 mM IPTG with expected size of 47 kDa. Recombinant Δ15 FAD was purified in two steps of chromatography: Hydrophobic Interaction Chromatography (HIC) and Ion Exchange Chromatography (IEX) with resin Butyl-S Sepharose 6 Fast Flow and SP Sepharose Fast Flow, respectively with the total protein yield of 1.28 mg/mL. The characterization of recombinant Δ15 FAD revealed desaturase activity of Δ15 FAD could produce α-linoleic acid at retention time of 22.3 with the composition of 32%. Characterization of semi-purified Δ15 FAD revealed the optimal temperature was 40 °C with 1 mM preferred substrate concentration of linoleic acid. The analysis of CD showed Δ15 FAD was made up of 51.8 % and 2.2 % of α-helix and β-sheet secondary structures, respectively. The predicted Tm value for Δ15 FAD was 49 °C. The three-dimensional (3D) structure of each recombinant protein was predicted using YASARA software and verified against validation tools; ERRAT2, Verify3D, and PROCHECK. Among the three FAD, Δ15 FAD structure displayed the best validation score. In conclusion, Δ6 FAD had been isolated and expressed in *E. coli* BL21 (DE3), whereas Δ12 FAD and Δ15 FAD had been successfully synthesized, overexpressed, purified and characterized to enhance the way towards understanding and modification of membrane protein.

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EKSPRESI GEN, PENCIRIAN DAN KAJIAN DALAM SILIKO TERHADAP ASID LEMAK DESATURASE DELTA

Oleh

NUR FARAH ANIS ABD HALIM

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Asid lemak desaturase (FAD) memangkinkan tindak balas penyahtepuan dengan memasukkan ikatan berganda ke dalam rantai asid lemak, menghasilkan asid lemak tak tepu. Walaupun asid lemak desaturase larut telah dikaji secara meluas dalam organisma hidup yang lebih tinggi, terdapat kajian yang sangat terhad tentang asid lemak desaturase membran kerana kesukaran menghasilkan desaturase rekombinan serta kecenderungan tinggi menjadi sel toksik. Asid lemak desaturase membran adalah menjadi keutamaan kerana ia memainkan peranan penting dalam sintesis asid lemak tak tepu, menawarkan faedah nutrisi kepada manusia. Dalam kajian ini, pelbagai asid lemak desaturase yang terdiri daripada asid lemak desaturase FAD Δ6, Δ12, dan Δ15 telah dipilih daripada organisma berbeza yang wujud dengan banyaknya untuk membuka laluan kepada pencirian. FAD Δ6 telah dipencilkan dan diamplifikasi secara PCR daripada *Anoxybacillus geotermalis,* diklon dalam vektor pGEX-4T1 dan diekspreskan dalam *E. coli* BL21 (DE3) dengan jangkaan amplikon pengekodan saiz 1104 bp untuk 368 asid amino yang diperoleh dengan enzim sekatan, *Bam*HI dan *Eco*RI. Rangka bacaan terbuka 1230 bp pengekodan asid lemak desaturase Δ12 untuk 410 asid amino daripada *Brassica napus* telah berjaya disintesis. Ia telah diklon dalam pET-51b(+) dengan enzim sekatan *Sal*I dan *Not*I, dan diekspreskan dalam *E. coli* BL21 (DE3) pada 37 °C dalam pecahan badan intrasel dan jasad inklusi apabila teraruh dengan 0.5 mM IPTG, dengan saiz dijangka 49 kDa. FAD Δ12 rekombinan berjaya ditulenkan dalam dua langkah kromatografi; Kromatografi Interaksi Hidrofobik (HIC) dan Kromatografi Pertukaran Ion (IEX) dengan resin *Butyl-S Sepharose 6 Fast Flow* dan *SP Sepharose Fast Flow*, masing-masing dengan jumlah hasil protein 1.55 mg/mL. Pencirian FAD Δ12 rekombinan mendedahkan aktiviti

FAD Δ12 boleh menghasilkan asid linoleik daripada asid oleik pada masa pengekalan 17.6 dengan komposisi 47%. Analisis dikreisme bulat (CD) menunjukkan FAD Δ12 terdiri masing-masing daripada 47.3% dan 0.9% struktur sekunder heliks-*α* dan lembaran-*β*. Nilai Tm yang diramalkan ialah 50.2 °C. FAD Δ15 telah disintesis daripada *Synechocystis* sp., *cyanobacterium* dan diklonkan dalam pET-51b(+) dengan enzim sekatan *Sal*I dan *Not*I dengan jangkaan saiz pengekodan sekitar 1155 bp untuk 385 asid amino. Protein diekspreskan dalam pecahan badan intrasel dan jasad inklusi dalam *E. coli* BL21 (DE3) pada 30 °C apabila teraruh dengan 1.0 mM IPTG dengan saiz dijangka 47 kDa. FAD Δ15 rekombinan telah separa ditulenkan dalam dua langkah kromatografi; Kromatografi Interaksi Hidrofobik (HIC) dan Kromatografi Pertukaran Ion (IEX) dengan resin *Butyl-S Sepharose 6 Fast Flow* dan *SP Sepharose Fast Flow*, dengan jumlah hasil protein 1.28 mg/mL. Pencirian FAD Δ15 rekombinan mendedahkan aktiviti FAD Δ15 boleh menghasilkan *α*–asid linoleik pada masa pengekalan 22.3 dengan kadar penukaran 32%. Pencirian FAD Δ15 yang telah separuh ditulenkan mendedahkan suhu optimum ialah 40 ° C dengan kepekatan substrat pilihan adalah 1 mM asid linoleik. Analisis CD menunjukkan FAD Δ15 terdiri masing-masing daripada 51.8% dan 2.2% daripada struktur sekunder heliks-*α* dan lembaran-*β*. Nilai Tm yang diramalkan untuk FAD Δ15 ialah 49 ℃. Struktur tiga-dimensi (3D) setiap protein rekombinan telah diramalkan menggunakan perisian YASARA dan disahkan menggunakan alat pengesahan; ERRAT2, Verify3D dan PROCHECK. Antara tiga FAD, struktur FAD Δ15 menunjukkan skor pengesahan terbaik. Kesimpulannya, FAD Δ6 telah dipencilkan dan diekspreskan dalam *E. coli* BL21 (DE3), manakala FAD Δ12 dan FAD Δ15 telah berjaya disintesis, diekspreskan, ditulenkan dan dicirikan untuk meningkatkan cara ke arah pemahaman dan pengubahsuaian protein membran.

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LIST OF ABBREVIATIONS

FAD	Fatty acid desaturase
NADP	Nicotinamide adenine dinucleotide phosphate ACP
phosphate ACP	Acyl-acyl carrier protein
CoA	Acyl-coenzyme A
SFA	Saturated fatty acids
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
GLA	Gamma-linolenic acid
DGLA	Dihomo-gamma linolenic acid
AA	Arachidonic acid
ALA	α-linolenic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
UFA	Unsaturated fatty acids
DNA	Deoxyribonucleic acid
PDB	Protein Data Bank
IPTG	Isopropyl β-d-1-thiogalactopyranoside
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis IEX
HIC	Hydrophobic Interaction Chromatography
GC-MS	Gas chromatography–mass spectrometry LB
FAMEs	Fatty acid methyl esters
A/H	Area/height
CD	Circular Dichroism
bp	Base pair
^{o}C	Degree Celsius
g	Gram
pH	Power of hydrogen
GRAVY	Grand average of hydropathy

RMS	Root mean square
L	Liter
mM	Millimoles
YASARA	Yet Another Scientific Artificial Reality Application
TEMED	Tetramethylethylenediamine

CHAPTER 1

INTRODUCTION

1.1 Introduction

There may be a preamble at the beginning of a chapter. The purpose may be to introduce the themes of the main headings. Membrane proteins, or more precisely, integral membrane proteins, contain hydrophobic surfaces that enable the proteins to bind tenaciously to lipid bilayers. Integral membrane proteins are defined as proteins that can be extracted from the lipid bilayer with an alkaline buffer or high concentrations of urea or salt. They consist of monotopic proteins, which bind to the membrane tightly but do not traverse it (Kongpracha et al., 2022). Bitopic proteins span the membrane only once, while polytopic proteins span the membrane several times. Based on the membrane-crossing secondary structure, there are two types of integral membrane proteins: α-helical transmembrane domain (TMD) proteins and β-barrel proteins. All membranes contain α-helical membrane proteins; however, only bacterial outer membranes, as well as mitochondria and chloroplasts outer membranes, contain β-barrel membrane proteins (Walther et al., 2009). Due to their low quantities and instability outside of lipid bilayers, these membrane proteins are difficult to examine using biochemical methods (César-Razquin et al., 2015).

Unsaturated fatty acids (UFA) are fatty acids with one or more double bonds in a variety of positions and configurations along the carbon backbone. Both monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were derived from UFA (Mthiyane and Hugo, 2019) through the desaturation reaction of fatty acids, which is catalyzed by the enzyme fatty acid desaturase (FAD) (Liu et al., 2021). This reaction requires molecular oxygen, NAD(P)H, an electron transport system (ferredoxin in NADPH reductase and ferredoxin from organisms, or cythochrome b_5 reductase and cytochrome b_5), and a terminal desaturase (Nakamura & Nara, 2004). FAD can be found universally as they present in all groups of organisms, including bacteria, fungi, plants, and animals (Los and Murata, 1998). The unsaturation of fatty acids in glycerolipids is essential for the proper functioning of biological membranes.

Thus, the introduction of an appropriate number of unsaturated bonds into the fatty acids of membrane glycerolipids decreases the temperature for the transition from the solid to the liquid-crystalline phase and provides the membrane with the necessary fluidity (Hazel, 1995). Soluble and membrane-

bound desaturases are the two types of FAD. The only soluble enzymes identified are the acyl carrier protein (ACP) desaturases, which are found primarily in the plastids of higher plants, whereas a large group of membrane-bound desaturases, including acyl-lipid and acyl-coenzyme A (CoA) desaturases, have been recognized on the endoplasmic reticulum and plastic membranes of prokaryotes, eukaryotes, and plants. They were said to have developed independently without being related to one another (Shanklin and Cahoon, 1998).

Membrane-bound desaturases in plants have three conserved histidine boxes and are divided into subfamilies with Δ3, Δ4, Δ5, Δ6, Δ7, Δ8, Δ9, Δ12, and Δ15 FAD (Shanklin and Cahoon, 1998; Sperling et al., 2003; Gao et al., 2009), converting saturated fatty acids (SFA) to MUFA and PUFA. In the pathway of FAD, Δ6 FAD, Δ9 FAD, Δ12 FAD and Δ15 FAD complement the pathway from saturated stearic acid to obtain product of α-linolenic acid (ALA). ALA has various properties for which it can be classified as essential fatty acids (Narinder et al., 2014). In contrast to soluble FAD, which has received extensive research, membrane FAD is still understudied, and has proven to be difficult to study due to their partially hydrophobic surfaces, flexibility, and lack of stability. The yield of membrane recombinant FAD protein is usually low, and inclusion body formation is also a serious problem. Literature of Δ9 FAD can be found abundantly and the study of Δ9 FAD has been studied considerably, even the structure of Δ9 FAD stearoyl-coA of mammalian (Bai et al., 2015) and steaoryl-coA of human have been elucidated (Wang et al., 2015) Nevertheless, for Δ6 FAD, Δ12 FAD and Δ15 FAD, limited studies were conducted.

In the present study, Δ6 FAD, Δ12 FAD, and Δ15 FAD, classified under acyl-lipid membrane-bound desaturase, have been cloned and expressed in *Escherichia coli (E. coli)* to facilitate recombinant protein and pave the way for structural characterization. Δ6 FAD, Δ12 FAD, and Δ15 FAD were chosen from different sources as they are available abundantly in different types of organisms accordingly. To the best of knowledge, there are still limited studies regarding acyl-lipid desaturases of membrane proteins. These three proteins were investigated as the biosynthesis pathway of FAD exhibited a variety of desaturases ranging from SFA to end products of UFA with three times desaturation. The main goal of this study was to obtain enough purified recombinant protein to open possibilities for characterization of the function, structure, and interactions of the proteins. Having purified FAD is essential as it can assist with structure elucidation and increase the production of PUFA, which crucial for human health and nutrition, and contribute to the understanding of the regulation of PUFA for biotechnological advances.

1.2 Problem statement

To date, genes of membrane FAD from numerous organisms have been published and expressed in various hosts, however, the technical difficulties in obtaining purified recombinant protein are still a major bottleneck. This is contrary to soluble FAD, which have been studied extensively. Nonetheless, information on purification and characterization of membrane FAD is still lacking, which the needs to have this information are crucial to open possibilities for characterization of the function, structure, and interactions of the FAD protein.

1.3 Hypothesis

By means of expression and purification of the gene, sufficient protein will be obtained for further biochemical and biophysical characterization. Hence, the characterization of protein and its function could be understood and provide required information for protein engineering.

1.4 Objectives

The main objective of this project is to express and characterize FAD from organisms and the specific objectives are listed below:

1. Cloning, expression, and structure prediction of Δ6 FAD gene in *E. coli.*
2. Cloning, expression, characterization, and structure prediction of Δ12 FAD gene in *E. coli.*
3. Cloning, expression, characterization, and structure prediction of Δ15 FAD gene in *E. coli.*

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