UNIVERSITI PUTRA MALAYSIA

MULTIPLE DOMAIN DELETION TOWARDS 5’-3’ POLYMERASE ACTIVITY OF DNA POLYMERASE I FROM Geobacillus sp. STRAIN SK72

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FBSB 2021 23

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By

WAQIYUDDIN HILMI BIN HADRAWI

Thesis Submitted to School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the degree of Master of Science

June 2021

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

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June 2021

Chairman : Raja Noor Zaliha Raja Abd. Rahman, D.Eng.
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DNA polymerases are members of family of enzyme that are essential in cellular replication and maintaining the genetic heredity of organisms. They are widely used in molecular research, especially the thermostable DNA polymerases, whereby their strong resistance to high temperatures are especially useful in DNA amplifications via the Polymerase Chain Reaction (PCR). DNA polymerase I had gained more attention in the protein-ligand interaction studies and its recent application in isothermal amplification. It is made up of three major domains that consist of a single polymerization domain and two additional exonuclease domains. Upon deletion of the N-terminal 5'-3' exonuclease domain, the enzyme is still able to demonstrate a full polymerase activity with an additional function used in isothermal amplification. However, the center 3'-5' exonuclease domain shows the absence of catalytic activity. The purpose of this inactive domain towards the DNA polymerase I is still unknown. Thus, in this study, a DNA polymerase I, namely, SK72 from *Geobacillus* sp. strain SK72 was used to understand the function of this disabled domain by studying the effect of domain deletion towards the polymerase activity. Three recombinant constructs were successfully developed based on the number of domains present. Each of the domain was identified based on the conserved regions through multiple databases such as Conserved Domain Search (CDS), InterPro Scan and Protein Families (Pfam). The regions were then analyzed to determine the appropriate first codon for each variant to prevent cutting important structures by using secondary structure prediction online tools including PSIPRED, Porter 4.0 and STRIDE. The 3D structure of each construct was predicted using YASARA software and verified against three validation tools namely ERRAT2, Verify3D, PROCHECK and QMEAN. All recombinant constructs were successfully expressed, and their proteins were purified in a single-step affinity chromatography prior to characterization. Variant SK72-Exo (large fragment without the 5'-3' exonuclease domain) showed similar optimum temperature and pH with wild-type SK72 (with all three domains) at 60 °C and pH 9, respectively,

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but exhibited the highest catalytic activity followed by SK72 and SK72-Exo2. It was also able to retain 80% of its activity at 60 ^{o}C and displayed the highest helix composition among all. Meanwhile, SK72-Exo2 (without both the exonuclease domains) had the lowest polymerase activity with an optimum temperature of 40 ^{o}C and expressed pH of 7. It was the least stable amongst the variants, showing a total inactivation at 50 ^{o}C. In conclusion, the removal of 5'-3' exonuclease domain had improved the polymerase activity and stability, while deleting both exonuclease domains had impaired the function of the polymerization domain. Thus, it is concluded that the 3'-5' exonuclease domain might be considered as a major structural domain instead of carrying any catalytic function.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMANSUHAN DOMAIN BERBILANG TERHADAP AKTIVITI POLIMERASE 5'-3' DNA POLIMERASE I DARIPADA *Geobacillus* sp. STRAIN SK72

Oleh

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DNA polimerase adalah anggota keluarga enzim yang penting dalam replikasi selular dan mengekalkan keturunan genetik organisma. Mereka digunakan secara meluas dalam penyelidikan molekul, terutama polimerase DNA termostabil di mana ketahanan kuatnya terhadap suhu tinggi sangat berguna dalam amplifikasi DNA melalui Reaksi Rantai Polimerase (PCR). DNA polimerase I mendapat perhatian lebih dalam kajian interaksi protein-ligan dan penerapannya baru-baru ini dalam amplifikasi isotermal. Ini terdiri daripada tiga domain utama yang terdiri daripada domain polimerisasi tunggal dan dua domain eksonuklease tambahan. Setelah penghapusan domain N-terminal 5’-3’ eksonuklease, enzim masih dapat menunjukkan aktiviti polimerase penuh dengan fungsi tambahan yang digunakan dalam amplifikasi isotermal. Walau bagaimanapun, domain 3’-5’ eksonuklease pusat menunjukkan ketiadaan aktiviti pemangkin. Tujuan domain tidak aktif ini terhadap DNA polimerase I masih belum diketahui. Oleh itu, dalam kajian ini, DNA polimerase I, iaitu SK72 dari *Geobacillus* sp. strain SK72 digunakan untuk memahami fungsi domain kurang upaya ini dengan mengkaji kesan penghapusan domain terhadap aktiviti polimerase. Tiga konstruk rekombinan berjaya dibangunkan berdasarkan jumlah domain yang ada. Setiap domain dikenal pasti berdasarkan kawasan terpelihara melalui beberapa pangkalan data seperti Pencarian Domain Terpelihara (CDS), InterPro Scan dan Protein Families (Pfam). Kawasan terpelihara kemudian dianalisis untuk menentukan kodon pertama yang sesuai untuk setiap varian untuk mengelakkan pemotongan struktur penting dengan menggunakan alat dalam talian ramalan struktur sekunder termasuk PSIPRED, Porter 4.0 dan STRIDE. Struktur 3D setiap konstruk diramalkan menggunakan perisian YASARA dan disahkan terhadap tiga alat pengesahan iaitu ERRAT2, Verify3D, PROCHECK dan QMEAN. Semua konstruk rekombinan berjaya diekspreskan dan proteinnya ditulenkan dalam satu langkah kromatografi keafinan sebelum pencirian. Varian SK72-Exo (fragmen besar tanpa domain eksonuklease 5’-3’’) menunjukkan suhu dan pH optimum yang serupa dengan SK72 jenis liar

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(dengan ketiga domain) masing-masing pada suhu 60 °C dan pH 9, tetapi menunjukkan aktiviti pemangkin tertinggi diikuti oleh SK72 dan SK72-Exo2. Ia juga dapat mengekalkan 80% aktivitinya pada suhu 60 °C dan menunjukkan komposisi heliks tertinggi di antara semua. Sementara itu, SK72-Exo2 (tanpa kedua-dua domain eksonuklease) mempunyai aktiviti polimerase terendah dengan suhu optimum 40 °C dan menyatakan pH 7. Ini adalah yang paling tidak stabil di antara varian, menunjukkan ketidakaktifan sepenuhnya pada 50 °C. Kesimpulannya, penghapusan domain eksonuklease 5'-3' telah meningkatkan aktiviti dan kestabilan polimerase, sementara menghapus kedua domain eksonuklease telah merosakkan fungsi domain polimerisasi. Oleh itu, dapat disimpulkan bahawa eksonuklease 3'-5' mungkin dianggap sebagai domain struktur utama dan bukannya menjalankan fungsi pemangkin.

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ACKNOWLEDGEMENTS

Foremost, I would like to thank Allah s.w.t for the strength and passion to complete this masters' project. Deepest gratitude to my supervisor and mother-in-lab, Prof. Dr. Raja Noor Zaliha Raja Abd. Rahman, for her continuous support in completing my research and thesis. Besides that, I would like to thank the rest of my thesis committees, Assoc. Prof. Dr. Mohd Shukuri Mohamad Ali, Dr. Fairolniza Mohd Shariff, and other principal investigators of Enzyme and Microbial Technology Research center (EMTech), Assoc. Prof. Dr. Adam Thean Chor, and Dr. Nor Hafizah Ahmad Kamaruddin for their guidance and thoughtful comments.

I thank my fellow lab mates in EMTech and colleagues in 12th residential college fellows for all their support and encouragement.

Finally, I would like to thank my family, the source of my strength and patient, for their understanding and prayers for me to complete my journey here.

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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

α	Alpha
β	Beta
°C	Degree Celsius
δ	Delta
ε	Epsilon
%	Percentage
^3H	Tritium
γ	Gamma
A_{600nm}	Optical density at wavelength 600 nanometer
µ	Micro
µL	Microliter
µm	Micrometer
µmoles	Micromoles
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic acid triphosphate
IMAC	Immobilized metal ion chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
g	Gram
kb	Kilobase
kDa	Kilo Dalton
L	Litre
M	Molar
Mg^{2+}	Magnesium ion

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$MgCl_2$	Magnesium chloride
Ni^{2+}	Nickel ion
OD_{280nm}	Optical density at wavelength 280 nanometre
OD_{600nm}	Optical density at wavelength 600 nanometre
Poly-His	Poly-histidine
PSI-BLAST	Position-Specific Iterated BLAST
rpm	Rotation per minute
u/mL	Unit per milliliter
u/mg	Unit per milligram
sp.	Species
SSB	Single strand binding protein
tRNA	Transfer ribonucleic acid

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CHAPTER 1

INTRODUCTION

1.1 Introduction

DNA polymerases are present in all types of organisms and are classified as one of the important elements in DNA replication (Garcia-Diaz & Bębenek, 2007; Choi, 2012). Basically, DNA polymerase does not replicate the DNA from scratch (de novo) instead, it is involved in the catalysis of the polymerization reaction of these duplexes by incorporating incoming nucleotides onto the complementary bases of the template strand (Alberts *et. Al*, 2002). There are various types of DNA polymerases, classified based on the size, shape, domain structure and sequence arrangement (Choi, 2012). However, they share similar structural frameworks and functions (Tripathi, 2010). The classification of DNA polymerases is primarily based on the highly conserved region in the core catalytic domain called polymerization domain. This domain is responsible for the 5'-3' polymerase activity that leads to the addition of free nucleotides to the growing strand. Some polymerases also include a single cutting enzyme on the N-terminal (Braithwaite et al., 1993; Hubscher et al., 2002).

DNA polymerase I or family A polymerase is one of the first DNA polymerases that was discovered and widely studied. The molecular structure is made up of three major domains namely, (i) 5′-3′ exonuclease domain; (ii) 3′-5′ exonuclease domain (absent in some enzymes); and (iii) 5'-3' polymerase domain (Pitrowski et al., 2019). The structural organization of the polymerase domain was divided into three subdomains called palm, fingers and thumb. The finger plays a role in holding the DNA template while the thumb appears to function in adding incoming nucleotide to the DNA strand. The polymerization reaction occurs within the palm subdomain by catalyzing the transfer of the phosphoryl group initiated by two-metal mechanisms. Meanwhile, the 3'-5' exonuclease domain is responsible for proof-reading activity (absent in some DNA polymerases), and the 5'-3' exonuclease domain serves to fill the gaps that exist in the Okazaki fragments at the lagging strand (Steitz et al., 1999; Joyce et al., 1994; Lovett et al., 2011).

In 1997, a new DNA polymerase I from *Geobacillus stearothermophilus* called *Bst*-DNA polymerase was successfully characterized and crystallized (Phang et al., 1995; Kiefer et al., 1998). The overall structure of the *Bst*-DNA polymerase was most similar to the Klenow fragment from *E. coli*, which is made up of only two major domains (3'-5' exonuclease domain and 5'-3' polymerase domain). This indicated that the *Bst*-DNA polymerase still able to retain its catalytic activity even though one of the major domains (5'-3' exonuclease domain) was deleted. In addition, the 3'-5' exonuclease domain that should carry a proofreading activity was disabled after performing exonuclease assay (Aliotta et al., 1996).

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Based on protein sequence analysis of the Bst-DNA polymerase with Klenow fragment, it was observed that *Bst*-DNA polymerase lack of conserved residues on the catalytic region of the 3'-5' exonuclease domain (Shevelev et al., 2002; Ishino et al., 1994; Aliotta et al., 1996). It was further verified through co-crystallization of the polymerase and its DNA duplexes that showed the absence of divalent metal ion interaction between the DNA template and the enzyme as metal ion that initiated the polymerization reaction (Kiefer et al., 1998).

The effect of truncation on enzymes is often investigated in order to understand the structure and function relationship by comparing the variant with its native form. Some variants may promote better characteristics, and some may diminish the catalytic activity. For instance, a study by Kamaruddin et al. (2014) removed four amino acid residues on the C-terminal tail of AT2 lipase from *Staphylococcus epidermidis,* which revealed an improvement in terms of protein crystallizability and anti-aggregation property. Meanwhile, Lamers et al**.** (2006) reported the structure organization of DNA polymerase III from *E. coli* that consists of a ten sub-unit complex with an N-terminal domain called Polymerase and Histidinol Phosphatase (PHP). However, this domain is presumed to be non-active but may involve in maintaining the structural integrity. A deletion study of a 60 N-terminal residue confirmed the role of the PHP domain as a major structural domain instead of a catalytic domain (Barros et al., 2013). Thus, it is hypothesized that the 3'-5' exonuclease domain in some DNA polymerase I might be a structural domain instead of catalytic domain (Kiefer et al., 1997).

In this study, a novel DNA polymerase I from *Geobacillus sp*., namely SK72 DNA polymerase that was previously isolated from the hot spring area at Sg. Klah, Perak, showed the existence of all three major domains. Furthermore, it revealed that the molecular structure was highly conserved with the DNA polymerase I from *Bacillus stearothermophilus*, whereby the 3'-5' exonuclease domain is non-functional (Aliotta et al., 1996; Pitrowski et al., 2019). To date, the role of this disabled exonuclease domain still remains unexplored; either its presence just as an inactive domain or might provide structural support towards the polymerization domain. Thus, this research aims is to understand the effect of multiple domain deletion in SK72 DNA polymerase and to perform a comparative study between these variants namely; SK72 (with all three major domains), SK72-Exo (large fragment without the 5'-3' exonuclease domain), and SK72-Exo2 (without both the exonuclease domain).

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1.2 Problem Statement

To date, there are no reports on the comparative analysis between the full-length of DNA polymerase I and its truncated enzyme available. Until now, the study on the removal 3'-5' exonuclease domain and its effect towards the polymerization domain is still unknown. Thus, this study aims to understand the structure-function relationship of the major domain in SK72 DNA polymerase with its core catalytic function as well as provide understanding in family A polymerases.

1.3 Hypothesis

It is postulated that the 3'-5' exonuclease domain is a major structural domain instead of carrying any catalytic activity. Thus, removing the 3'-5'exonuclease domain might impact the polymerase activity.

1.4 Objectives

The main objective of this research was o compare the effect on multiple domain deletion in SK72 DNA polymerase through biochemical and biophysical characterization. The specific objectives of this study were as follows.

i. To construct the SK72 DNA polymerase and its variants (SK72-Exo and SK72-Exo2) through *in-silico* studies.
ii. To clone and express the SK72 DNA polymerase and its variants
iii. To purify and characterize the SK72 DNA polymerase and its variants.

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