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IN-SILICO STRUCTURAL ANALYSIS AFFECTING THERMOSTABILITY IN RECOMBINANT PSYCHROPHILIC CHITINASE (CHI II) FROM *Glaciozyma antarctica* PI12

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A thesis submitted in fulfilment of the
requirement for the award of the degree of
Master of Engineering (Bioprocess)

Faculty of Chemical and Energy Engineering
Universiti Teknologi Malaysia

JANUARY 2017

The day has finally come. When I can say it out loud
'I made it'

To *mama & bapak*,
this is for you

ACKNOWLEDGEMENT

Alhamdulillah, all praises to Allah, Whom I am grateful for His guidance. Without His grant, this thesis could be possible. His divine and complex creations never failed to amaze me. I am thankful to my supervisor, Prof Dr. Rosli Md Illias for his encouragement and guidance through all these years. Not to forget, Encik Yaakop for the technical support provided. I would like to express my gratitude to Ministry of Science, Technology and Innovation Malaysia for the scholarship and research funding.

My utmost appreciation to *mama* and *bapak*, my sibling, Liyana, Luqman and Amirah for their endless love, encouragement and prayer. To my best friends Anita, Hazwani and Suhailis for their endless motivation and support. My deep appreciation also goes to my labmates, for your help, knowledge and unwavering support through ups and downs. Thanks for this friendship. I wish you guys luck. Not to forget, Dr Aizi Nor Mazila. Thank you so much for your knowledge and guidance in my early journey. I also wish to show my appreciation to those who directly or indirectly involved in finishing this thesis.

To all of you, thank you so much. I am blessed

ABSTRACT

Cold-adapted enzymes are significant with structure flexibility and high catalytic activity at low temperature. High structural flexibility could be due to combination of several features such as weak intramolecular bonds, decreased compactness of hydrophobic core and reduced number of proline and arginine residues. However, to compensate the structural flexibility, cold-adapted enzymes are also thermolabile which causes them to be easily inactivated at elevated temperature**.** Therefore, it would be more interesting and beneficial if more stable cold-adapted enzymes are produced to fulfill the industrial needs. In this study, a novel cold-adapted chitinase (CHI II) from *Glaciozyma antarctica* PI12 was rationally designed to improve their thermostability thus make them more resistant to increased temperature. Four CHI II mutants were designed through rational design named as A157Q, I134P, mutant Loop and Y257R by manipulating the structural hydrophobicity, introduction of proline in the loop regions, introduction of arginine salt bridges and loop shortening. Mutant Loop was designed by removing 9 residues in loop regions thus makes loop involved became shorter. Stability of all mutants was first predicted through a computational approach where all structures were subjected to 10 ns molecular dynamics simulation at three temperatures; 273 K, 288 K and at 300 K. Based on the simulation, it was found that mutants I134P, mutant Loop and Y257R exhibited structural stability at 300 K. This conclusion was made based on low and stable root-mean square deviation (RMSD) value at 300 K in comparison to RMSD values at 288 K and 273 K. Low RMSD values indicated mutant structure experienced low structural deviation throughout the simulation. Besides, this observation is correlated with reduction of structure compactness (radius of gyration), reduced solvent accessible surface area and increased numbers of hydrogen and salt bridges. However, mutant A157Q experienced structure destabilization at 300 K. Substitution of helix-preferred residue, alanine with a thermolabile residue, glutamine had caused A157Q structure become loosely packed at 300 K indicating a thermal denaturation. To support the theoretical model, CHI II and all mutants were then cloned into *Pichia pastoris* expression vector pPICZαC and expressed in *P. pastoris* (GS115).

ABSTRAK

Enzim tahan sejuk adalah dikenali dengan struktur yang fleksibel serta aktiviti bermangkin yang tinggi pada suhu rendah. Fleksibiliti struktur adalah disebabkan oleh kombinasi beberapa ciri seperti ikatan intramolekul yang lemah, penurunan kepadatan teras hidrofobik dan pengurangan sisa prolina dan arginina. Walau bagaimanapun, untuk menebus kembali fleksibiliti strukturnya, enzim tahan sejuk bersifat termolabil yang menyebabkannya mudah untuk ternyahaktif pada suhu lampau tinggi. Oleh itu, ianya sangat menarik dan bermanfaat jika enzim tahan sejuk yang lebih stabil dapat dihasilkan bagi memenuhi keperluan industri. Dalam kajian ini, enzim kitinase tahan sejuk (CHI II) dari organisma *Glaciozyma antarctica* PI12 telah direkabentuk secara rasional untuk meningkatkan tahap termostabilnya dan menjadikan CHI II lebih tahan kepada peningkatan suhu. Empat mutan CHI II telah di rekabentuk melalui rekabentuk rasional yang dinamakan sebagai A157Q, I134P, Gelung mutan dan Y257R dengan mengubah suai kehidrofobikan struktur, memperkenalkan prolina di kawasan gelung, memperkenalkan titian garam arginina serta pemendekan gelung. Gelung mutan telah direkabentuk dengan pemotongan 9 sisa di kawasan gelung menyebabkan gelung yang terlibat menjadi semakin pendek. Kestabilan kesemua mutan diramalkan terlebih dahulu melalui pendekatan pengkomputeran di mana kesemua struktur mutan tertakluk kepada simulasi 10 ns dinamik molekul yang dijalankan pada tiga suhu iaitu pada 273 K, 288 K dan 300 K. Berdasarkan simulasi yang dijalankan, mutan I134P, gelung mutan dan Y257R menunjukkan nilai sisihan punca min kuasa dua (RMSD) yang rendah dan stabil pada suhu 300 K, jika dibandingkan dengan nilai RMSD pada 288 K dan 273 K. Nilai RMSD yang rendah menggambarkan struktur mutan telah mengalami sisihan berstruktur rendah keseluruhan simulasi dijalankan. Sebaliknya, pemerhatian ini berkorelasi dengan penurunan kepadatan struktur (jejari legaran), penurunan luas permukaan boleh capai pelarut dan peningkatan ikatan hidrogen serta titian garam. Walau bagaimanapun, mutan A157Q mengalami penurunan kestabilan struktur pada 300 K. Penggantian sisa pilihan heliks, iaitu alanina dengan sisa termolabil, glutamina telah menyebabkan struktur A157Q menjadi longgar pada suhu 300 K menandakan penyahaslian terma. Untuk menyokong model teori ini, CHI II dan kesemua mutan telah diklon ke dalam vektor ekspresi *Pichia pastoris* pPICZαC dan dinyatakan dalam *P. pastoris* (GS115).

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LIST OF SYMBOLS AND ABBREVIATIONS

%	-	percent
*	-	asterisk
.top	-	Topology file
~	-	About
>	-	Greater
≥	-	Greater or equal to
°	-	Degree
°C	-	degree celcius
½	-	Half
3D	-	Three dimensional
A	-	Alanine
Å	-	Angstrom
Arg or R	-	Arginine
Asn	-	Asparagine
Asp or D	-	Aspartic acid/aspartate
BD	-	Binding domain
C. congregates	-	*Coprinellus congregates*
C. immitis	-	*Coccidioodes immitis*
CAZy	-	Carbohydrate Active Enzyme database
CBD	-	Chitin binding domain
CID	-	Chitinase insertion domain
Cl^-	-	Chloride ion
Cys	-	Cysteine
Cα	-	Carbon alpha

DNA	-	Deoxyribonucleic acid
DOPE	-	Discrete optimized protein energy
DSSP	-	Dictionary of secondary structure prediction
EC	-	enzyme commission
F	-	Phenylalanine
G. antarctica	-	*Glaciozyma Antarctica*
GH	-	Glycosyl hydrolase
GlcN	-	Glucosamine
GlcNAc	-	N-acetylglucosamine
Glu or E	-	Glutamic acid/glutamate
Glu or Q	-	Glutamine
Gly or G	-	Glycine
GPI	-	Glycosylphosphatidylinositol
H	-	Hydrogen
H. atriviridis	-	*Hypocrea atroviridis*
His or H	-	Hisitidne
ID	-	Identifier
Ile or I	-	Isoleucine
K	-	Kelvin
kDa	-	Kilodalton
kJ/mol	-	kilojoule per mol
L	-	Leucine
Lys or K	-	Lysine
LysM	-	Lysin motif
MD	-	Molecular dynamics
MSA	-	Multiple sequence alignment
N	-	Nitrogen
N		Asparagine
Na^+	-	Sodium ion
Nm	-	Nanometer
Ns	-	Nanoseconds
O	-	Oxygen
PDB	-	Protein data bank

pKa	-	Acid dissociation constant
PME	-	Particle mesh ewald
Pro or P	-	Proline
Rg	-	Radius of gyration
RMSD	-	Root mean square deviation
RMSF	-	Root mean square fluctuation
S. cerivisae	-	*Saccharomyces cerivisae*
SASA	-	Solvent accessible surface area
SCP	-	Single cell protein
Ser or S	-	Serine
sp.	-	Species
SPC	-	Simple point charge
T	-	Threonine
T. atroviride	-	*Trichoderma atroviride*
T. aurantiacus	-	*Thermoascus aurantiacus*
T. lanuginosus	-	*Thermomyces lanuginosus*
TIM	-	Triosephosphate isomerase
T_m	-	Midpoint temperature
Tyr orY	-	Tyrosine
V	-	Valine
VMD	-	Visual molecular dynamic
α	-	Alpha
β	-	Beta
γ	-	Gamma
φ	-	Phi
ψ	-	Psi
µg/µl	-	Microgram per microliter
µl	-	Microliter
µmol	-	Micromole
BMGY	-	Buffered glycerol complex medium
BMMY	-	Buffered methanol complex medium
Bp	-	Base pair
EDTA	-	Ethylenediaminetetraacetic acid

LB	-	Luria Bertani
M	-	Molarity
ml	-	Milliliter
mM	-	Millimole
OE	-	Overlapping extension
P. pastoris	-	*Pichia pastoris*
PCR	-	Polymerase chain reaction
U	-	Unit activity
V	-	Voltage
v/v	-	Volume over volume
YPD	-	Yeast extract peptone dextrose
YPDS	-	Yeast extract peptone dextrose sorbitol
zeo^R	-	Zeocin resistance

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Earth's surface is dominated by low temperature regions including polar region, mountains top and oceans which cover 80% of the biosphere where temperatures never exceed 5°C. Low temperatures are known to place severe physicochemical constraints on cellular function by negatively influencing cell integrity, water viscosity, solute diffusion rate, membrane fluidity, enzyme kinetics and macromolecular interactions (Marx *et al*., 2004). However, despite most other species cannot grow at this low temperature regions, an extremophiles known as psychrophiles are able to survive and inhabit this region. Then the question arises: how can psychrophiles survive, let alone thrive under this harsh conditions? The answer is, psychrophiles evolved and adapted to their environments by developing unique mechanisms to keep their cellular components stable and active.

The ability of psychrophiles to survive in cold regions is therefore dependent on numbers of adaptive strategies to successfully counteract those low temperature constraints (D'Amico *et al*., 2006). One of the strategies is by producing the cold-adapted enzymes that able to perform their catalysis efficiently under these extreme environmental conditions (D'Amico *et al*., 2006). For these reasons, cold-adapted enzymes have been considered as biotechnological potential due to their ability to perform catalysis at low temperatures thus offering advantages in the

environmental application and energy savings in industrials processes (Gianese *et al*., 2001).

While other enzymes are subject to cold denaturation and suffered the loss of activity at low temperatures, cold-adapted enzymes are resistant to cold denaturation with efficient catalytic activity. Their survival is correlated with their structural flexibility that was believed as a compensation for the freezing effect in cold habitats (Johns & Somero 2004). Structures flexibility of cold-adapted enzymes is the result of combination of several features such as increased numbers of hydrophobic side chains that are exposed to the solvent, a decrease in the compactness of hydrophobic core, a higher number of glycine and lysine residues, a reduced number of proline and arginine residues and weakening of intramolecular bonds (Rodrigues & Tiedje 2008). However, because of their structural flexibility, cold-adapted enzymes become less stable and also thermolabile which cause them to denature at elevated temperature (Siddiqui & Cavicchioli 2006).

Therefore, cold-adapted enzymes are often engineered either through rational design or directed evolution to improve its thermostability. Thermostability is defined as improved long-term survival under mild conditions and increased ability to remain active under harsh industrial condition but still retains its catalytic efficiency (Wijma *et al*., 2013). In this study, a cold-adapted chitinase named as CHI II was used as the subject understudied. Chitinase (EC 3.2.2.14) are categorized under glycosyl hydrolases (GH) family and can be found in wide range of organisms such as bacteria, fungi, yeasts, plants and mammals. Capabilities of chitinase to hydrolyse chitin to a low molecular weight chitooligomers cause them to have broad potential in industrial, agricultural and medicinal functions (Dahiya *et al*., 2006; Liu *et al*., 2013; Patil *et al*., 2000; Park & Kim 2010; Khan *et al*., 2015).

CHI II was previously isolated from *Glaciozyma antarctica* PI12 and it's three-dimensional (3D) structures had been modeled by Ramli *et al*. (2011, 2012). Based on the structure analysis and primary sequence analysis, several characteristics related to cold adaptations were found in CHI II. CHI II was identified to have less number of salt bridges and arginine residues, increase in surface hydrophobicity and reduced

number of hydrogen bonds (Ramli *et al*. 2012). These characteristics were proved to be related to structural flexibility of CHI II which causes CHI II to be thermolabile and could not withstand elevated temperature and harsh environment. In addition to the wide potential of chitinases in industrial application and biotechnological application, it is best for CHI II to be engineered to improve their thermostability. Based on the information obtain from previous study on amino acid affecting thermostability of cold adapted chitinase and based on comparison studies between mesophilic, psychrophilic and thermophilic enzymes, rational design was used to design CHI II mutants (Mavromatis *et al.*, 2003; Siddiqui & Cavicchioli 2006). Therefore, four mutants of CHI II will be designed through rational design and the effect of the mutation will be studied using an *in-silico* approach. In particular, the mutant's structure stability will be studied through molecular dynamic (MD) simulation at three temperatures: 273 K, 288 K and 300 K. This is an indicator of CHI II mutants performance as it reflects the ability of the mutants to perform under conditions relevant to an industrial process where enzyme is continually affected by temperature elevation.

1.2 Problem Statement

About 80 000 metric tons biomass waste of marine invertebrate were produced every year and it was predicted that the oceans will be depleted of chitin if this insoluble biomass is not converted into simple and recyclable material (Patil *et al*., 2000). Capabilities of cold-adapted chitinases to have high catalytic efficiency and high flexibility (low stability) at low temperatures allows them to offer several novel opportunities in industrial application. Because of their inherent flexible structure, cold-adapted chitinase was correlated to be thermolabile as their reaction rates decrease when the temperature increases. Hence, this condition becomes a limitation for cold-adapted chitinase to be used in industrial application. Thus, production of cold-adapted enzymes chitinase with desired thermostability become an important aspect of industrial application which could also help to overcome chitin depletion. This can be achieved through mutagenesis of cold-adapted chitinase to improve its thermostability without compromising its structurally dependent cold-adapted properties (Cesarini *et al*., 2012).

1.3 Objectives

The main objective of this study is to analyse the effect of amino acids substitution, loop shortening and introduction of the salt bridge in the non-catalytic region on CHI II thermostability through *in-silico* approach.

1.4 Scopes of Study

The scope of this study are:

a) Design four CHI II mutants through rational design.
b) Construction of four mutants three-dimensional (3D) structures using mutagenesis plugin in PyMOL and homology modeling by using Modeller.
c) Performing the Molecular Dynamics (MD) simulation of CHI II and its four mutants at three different temperatures; 273 K, 288 K and 300 K.
d) Performing comparative trajectories analysis on CHI II and its mutants to study the effect of mutation on CHI II thermostability.

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APPENDIX A

APPENDIX A: TUTORIAL FOR MD SIMULATION

1) Process the pdb file withpdb2gmx

pdb2gmx –ignh –f inputabf.pdb –o abf.pdb –p abf.top –water spce

Pdb2gmx command converts the pdb file to a gromacs file and write the topology.

Points to ponder:

- What is the total mass of your protein?
- What is the total charge of your protein?
- Open the topology file (abf.top) using Gedit, see how the force define protein

2) Set-up box for simulation

editconf –bt cubic –f abf.pdb –o abf_bsolv.pdb –d 2.0

Editconf specify the simulation box. '-d' sets the dimension of the box 2.0 nm (20A). It should at no less than 0.9 nm for most system.

3) Solvate the box

genbox –cp abf_bsolv.pdb –cs spc216.gro –o abf_bion.pdb –p abf.top

Points to ponder:

- Does the size of your box change after the solvation?
- How many SOL molecules were added into your simulation box?
- Any change to your topology file?

4) Neutralize the protein system

grompp –f ion.mdp –c abf_bion.pdb –p abf.top –o ion.tpr –maxwarn 5

genion –s ion.tpr –o abf_b4em.pdb –nname CL (–pname NA) –nn 2 (–np 2) –p abf.top –g ion.log

Type "13" for SOL

Points to ponder:

- How many NA and CL had been added into the system?
- Is there any charge in the topology file?
- What do the flags used along the genion?

5) Energy Minimization

grompp –f em.mdp –c abf_b4em.pdb –p abf.top –o em.tpr –maxwarn 5

mdrun –v –deffnm em

Points to ponder:

- What do –v and –deffnm mean?
- How many steps does the system take to converge?
- How many output files are there?

The output will be em.gro. If the F_{max} did not converge, repeat the step by changing the input and output file (em.mdp file did not change). Take output from first step (em.gro) as an input for second step (after '-c' command). The second step output will be em2.tpr (after '-o' command). Run the simulation by naming em2 at mdrun step.

6) Position Restrained Molecular Dymanics (equilibration)

grompp –f pr.mdp –c em.gro –p abf.top –o pr.tpr –maxwarn 5

Once the pr.tpr is generated successfully, run the position restrained MD

mdrun –v –deffnm pr

Points to ponder:

- Is there any note/warning when grompp is pre-processing the pr.mdp?

7) Convert Gromacs File

editconf –f file.gro –o file.pdb

8) First Evaluation

a) First evaluate the system and see the water molecules had been equilibrated or not

b) Compute the RMSD of the protein backbone and plot several graphs

g_rms –f pr.trr –s pr.tpr –o rmsd_pr

- Examine using GRACE. xmgrace rmsd_pr.xvg
- For least square fit and RMSD calculation, select group 4 (Backbone)

- The program will generate a plot for RMSD over time

c) Examine the temperature:

g_energy –f pr.edr –o temperature_pr

- Select ‘14’ (Temperature)
- Examine using GRACE. *xmgrace temperature_pr.xvg*

d) Use g_energy to plot density_pr.xvg and pressure_pr.xvg, use xmgrace command to plot the graph.

e) System had been equilibrated and may proceed to the production stage when:

a. The temperature plot stabilized/constant at 300K
b. The average reading for density_pr.xvg and pressure_pr.xvg are 1000 kg/m^3 and 1.05 bar respectively

9) Production Stage

grompp –f md.mdp –c pr.gro –p abf.top –o md.tpr –maxwarn 5

mdrun –v –deffnm md

10) Trajectories analysis

Time evolving coordinates of a system are called trajectories. Trajectory files (*.trr) are normally binary files that contain several sets of coordinates for the system.

a) Compress the trajectory

trjconv –f md.trr –s md.tpr –o md.xtc –pbc nojump

b) Analyse the energy output (same for potential energy, kinetic energy and total energy)

g_energy –f md.edr –o xxx and plot *xmgrace –nxy xxx.xvg,*

c) Measure radius of gyration and select ‘4’ (Backbone).

g_gyrate –f md.trr –s md.tpr –o abf_gyrate.xvg

d) Measure RMSD of the structure by and select ‘4’ (Backbone).

g_rms –s md.tpr –f md.trr –dt 10 –o md_rmsd.xvg

e) Compare RMSD to the NMR structure and select ‘4’ (Backbone)

g_rms –s em.tpr –f md.trr –o abf_rmsd.xvg

f) RMS fluctuation of atom positions and select ‘3’ (C-alpha).

g_rmsf –s md.tpr –f md.trr –b 200 –e 1000 –o abf.rmsf

g) RMSF to compute average structures and select ‘1’ (Protein).

g_rmsf –s md.tpr –f md.trr –b 800 –e 1000 –o abf_xvg.pdb

h) Analyse the secondary structure of model by and select ‘1’ (Protein).

do_dssp –s md.tpr –f md.trr –o abf_ss.xpm –dt 10