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CALCIUM-INDUCED FOLDING AND STABILIZATION OF COLD-ACTIVE RTX LIPASE FROM Pseudomonas sp. STRAIN AMS8

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By

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

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CALCIUM-INDUCED FOLDING AND STABILIZATION OF COLD-ACTIVE RTX LIPASE FROM *Pseudomonas* sp. STRAIN AMS8

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June 2020

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Repeat-in-Toxin (RTX) represents a broad family of protein produced by Gram-negative bacteria. RTX protein consists of RTX parallel *β*-roll motif repeat structure. Previously, AMS8 lipase from Antarctic *Pseudomonas fluorescens* strain AMS8 was classified as RTX lipase. The previous study has been reported the Ca^{2+} ions play a role in the formation of RTX parallel *β*-roll motif repeat structure and involve in the folding and stabilization of many RTX protein. However, the contributions of Ca^{2+} ions towards the folding and stabilization of AMS8 lipase have not been understood. It is hypothesized that the Ca^{2+} ions induce the formation of RTX parallel *β*-roll motif repeat structure and involve in the folding and stabilization of AMS8 lipase. Thus, this research aimed to examine the influence of Ca^{2+} ion towards the activity, folding and stabilization of AMS8 lipase through the *in-silico* approach and various biophysical characterizations.

AMS8 lipase contains six Ca^{2+} ions (Ca1, Ca2, Ca3, Ca4, Ca5 and Ca6) and RTX parallel *β*-roll motif repeat structures. *In-silico* studies were done to analyze the structural conformational changes of the AMS8 lipase structure using molecular docking and molecular dynamics (MD) simulation. As a result, metal ion docking analysis gives high binding energy, especially for Ca4 and Ca5. To further analyze the function of each Ca^{2+} ions, MD simulation was performed. The removal of Ca3, Ca4 and Ca5 caused the AMS8 lipase structure to become unstable and unfolded. These suggested that Ca3, Ca4 and Ca5 were involved in the stabilization and folding of the RTX parallel *β*-roll motif repeat structure.

AMS8 lipase activity was increased in the presence of $CaCl_2$, where the optimum $CaCl_2$ concentration was detected at 80 mM. To further confirm the contribution of Ca^{2+} ion, various biophysical characterizations using circular dichroism (CD), fourier-transform infrared (FTIR), intrinsic and extrinsic fluorescence, dynamic light scattering (DLS) and isothermal titration calorimetry (ITC) were performed. The far-UV CD and FTIR analyses suggested that the secondary structure content was improved with the addition of $CaCl_2$. Intrinsic and extrinsic fluorescence analysis showed that the presence of $CaCl_2$ increased protein folding and compactness. DLS analysis suggested the AMS8 lipase became aggregated at a high concentration of $CaCl_2$. The binding constant (K_d) value from the ITC analysis proved that the Ca^{2+} ion was tightly bound to the AMS8 lipase.

In conclusion, *in-silico* approach and various biophysical characterizations revealed that Ca^{2+} ions play essential roles in the activity, folding and stability of the AMS8 lipase. Furthermore, Ca^{2+} ions also induced the folding of the RTX parallel *β*-roll motif repeat structure and played a crucial role in the folding and stabilization purposes of the whole AMS8 lipase structure.

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ARUHAN KALSIUM KEPADA PENGLIPATAN DAN PENSTABILAN LIPASE RTX AKTIF SEJUK DARIPADA *Pseudomonas* sp. STRAIN AMS8

Oleh

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Repeat-in-Toxin (RTX) mewakili keluarga besar protein yang dihasilkan oleh Gram-negatif bakteria. RTX protein terdiri daripada struktur RTX selari *β*-roll motif ulangan. Sebelum ini, AMS8 lipase daripada Antartika *Pseudomonas fluoresens* strain AMS8 dikelaskan sebagai RTX lipase. Kajian sebelumnya telah melaporkan bahawa ion Ca^{2+} berperanan dalam pembentukan struktur RTX selari *β*-roll motif ulangan dan terlibat dalam lipatan dan penstabilan pelbagai protein RTX. Walau bagaimanapun, sumbangan ion-ion Ca^{2+} terhadap lipatan dan penstabilan AMS8 lipase belum difahami. Ia dihipotesiskan bahawa ion-ion Ca^{2+} mengaruh struktur RTX selari *β*-roll motif ulangan dan terlibat dalam lipatan dan penstabilan AMS8 lipase. Oleh itu, kajian ini bertujuan untuk mengkaji pengaruh ion Ca^{2+} terhadap aktiviti, lipatan dan penstabilan AMS8 lipase melalui pendekatan in-siliko dan pelbagai pencirian biofizik.

AMS8 lipase mengandungi enam ion Ca^{2+} (Ca1, Ca2, Ca3, Ca4, Ca5 dan Ca6) dan struktur RTX selari *β*-roll motif ulangan. Kajian in-siliko dilakukan untuk menganalisis perubahan konformasi struktur AMS8 lipase menggunakan molekular dok dan simulasi molekular dinamik (MD). Keputusannya, analisis dok logam ion memberikan tenaga pengikatan yang tinggi, terutama untuk Ca4 dan Ca5. Bagi menganalisis fungsi setiap ion-ion Ca^{2+}, simulasi MD dilakukan. Penyingkiran Ca3, Ca4 dan Ca5 menyebabkan struktur AMS8 lipase menjadi tidak stabil dan terbuka lipatan. Ini menunjukkan bahawa Ca3, Ca4 dan Ca5 terlibat dalam penstabilan struktur RTX selari *β*-roll motif ulangan.

Aktiviti AMS8 lipase meningkat dengan kehadiran $CaCl_2$ di mana kepekatan $CaCl_2$ optimum dikesan pada 80 mM. Bagi mengesahkan sumbangan Ca^{2+} ion,

pelbagai pencirian biofizik menggunakan circular dichroism (CD), fourier-transform infrared (FTIR), intrinsic and extrinsic fluorescence, dynamic light scattering (DLS) dan isothermal titration calorimetry (ITC) telah dilaksanakan. Analisis far-UV CD dan FTIR mencadangkan bahawa kandungan struktur sekunder bertambah baik dengan penambahan $CaCl_2$. Analisis intrinsic and extrinsic fluorescence menunjukkan bahawa kehadiran $CaCl_2$ meningkatkan lipatan dan ketumpatan protein. Analisis DLS mencadangkan bahawa AMS8 lipase menjadi agregat pada kepekatan $CaCl_2$ yang tinggi. Nilai binding constant (K_d) dari analisis ITC membuktikan bahawa ion Ca^{2+} terikat kuat pada AMS8 lipase.

Sebagai kesimpulan, pendekatan in-siliko dan pelbagai pencirian biofisik mendedahkan bahawa ion Ca^{2+} memainkan peranan penting dalam aktiviti, lipatan dan kestabilan AMS8 lipase. Tambahan pula, ion Ca^{2+} juga mendorong kepada pembentukan struktur RTX selari *β*-roll motif ulangan dan memainkan peranan penting dalam penglipatan dan penstabilan keseluruhan struktur AMS8 lipase.

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
µL	Microliter
µm	Micrometer
µmoles	Micromoles
Å	Angstrom
A_{600nm}	Optical density at wavelength 600 nanometer
ADP	Adenosine diphosphate
ATP	Adenosine 5'-triphosphate
bp	Base pair
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
g	Gram
Kb	Kilobase
kDa	Kilodalton
L	Litre
M	Molar
mg	Milligram
mL	Millilitre
N	Normality
ns	Nanosecond
Rpm	Revolutions per minute
sp.	Species
U	Unit
V	Volt
v/v	Volume over volume
w/v	Weight over volume
Zn^{2+}	Zinc ion

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Cold-active enzymes are active at low temperature and it can sustain the dynamics and flexibility of their active site at low temperatures (Latip et al., 2016). Cold-active enzymes have attracted much attention as biocatalysts due to their capacity to resist unfavorable reaction conditions in the industrial process (Hamid & Mohiddin, 2018). Cold-active enzymes including lipases are valuable in the biotechnology industry to reduce process times for low-temperature processes, conserve energy costs, minimize the enzyme concentration needed, prevent undesired chemical transformations, and prevent the loss of volatile compounds (Kuddus, 2014). Even though the lipase group contributes less than 10 % of the global enzyme market, it assists an extensive range of industrial purposes including food and oil processing, detergents and pharmaceutical end-users (Guerrand, 2017).

Previously, cold-active RTX lipase (AMS8 lipase) belongs to the I.3 family was isolated from Antarctic *Pseudomonas.* A lipase gene named *LipAMS8* was successfully isolated from strain AMS8, cloned, sequenced and overexpressed in *Escherichia coli* (Ali et al., 2013a). AMS8 lipase was classified as RTX (Repeat-in-Toxin) lipases due to the presence of RTX nanopeptide repeat sequence which produces the RTX parallel *β*-roll motif repeat structure at the C-terminal. These RTX nonapeptides repeat sequence form a number of binding sites for Ca^{2+} ions (Linhartova et al., 2010). In the absence of Ca^{2+} ion, these RTX parallel *β*-roll motif repeat structures seem to be mostly disordered and may disturb the entire stabilization and folding of protein structure (Gupta & Rathi, 2004; Sharma et al., 2001).

AMS8 lipase predicted structure consists of metal ion including one Zn^{2+} and six Ca^{2+} ions. The C-terminal consists of 3 RTX parallel *β*-roll motif repeat and 3 of the Ca^{2+} ion are bound to the RTX parallel *β*-roll motif repeat structure. Past studies have suggested that the RTX parallel *β*-roll motif repeat structure is responsible for internal chaperones, receptor binding domains and enhancers of the secretion process (Lilie et al., 2000). Even though the exact function of the RTX parallel *β*-roll motif repeat structure remains obscure, the presence of Ca^{2+} ion plays an essential role since it is relevant for both folding and stability of many protein domains (Stigler et al., 2011).

1.2 Problem statement

An earlier study proposed that lipase from Antarctic *Pseudomonas fluorescens* strain AMS8 was classified as RTX lipase. AMS8 lipase consists of RTX parallel *β*-roll motif repeat structure and Ca^{2+} ions. A previous study has been reported the Ca^{2+} ions affect the formation and stabilization of RTX parallel *β*-roll motif repeat structure and are involved in the folding and stabilization of many RTX proteins. However, the contributions of six Ca^{2+} ions toward folding and stabilization of AMS8 lipase not been understood.

1.3 Research objectives

Investigations were carried out with a combination of *in-silico* study and biophysical approaches to explore the effect of Ca^{2+} ion towards the activity, folding and stabilization of the cold-active RTX lipase from *Pseudomonas fluorescens* strain AMS8. Hence, the research was undertaken with the following sub-objectives:

1. To investigate the function of Ca^{2+} ion towards the structural conformation and stabilization of the AMS8 lipase structure and the RTX parallel *β*-roll motif repeat structure via the *in- silico* approach.

2. To characterize the role of Ca^{2+} ion towards the activity and folding of AMS8 lipase via various biophysical approaches.

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CHAPTER 5

CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

5.1 Conclusion

AMS8 lipase from *P. fluorescence* strain AMS8 belonged to the RTX lipases group since it contained RTX parallel *β*-roll motif repeat structures at the C-terminal. AMS8 lipase contained six Ca^{2+} ions. The Ca^{2+} ions were bound to the catalytic and non-catalytic domain of the structure, including the RTX parallel *β*-roll motif repeat structures. The *in-silico* study showed the removal of Ca^{2+} ions caused the destabilization and unfolding of the AMS8 lipase structure, especially at the non-catalytic domain, including the RTX parallel *β*-roll motif repeat structure. This showed that the contribution of calcium-binding performed a vital function in sustaining the structural integrity of AMS8 lipase. Besides, AMS8 lipase activity increased with the presence of Ca^{2+} ions. The evidence from lipase activity was further analyzed using various biophysical characterizations. The biophysical characterization analyses (Far-UV CD spectra, FTIR spectroscopy, fluorescence spectroscopy, DLS and ITC) revealed that the presence of Ca^{2+} ions improved the secondary structure and made the folding of AMS8 lipase better compared to untreated AMS8 lipase (without Ca^{2+} ion). Furthermore, Ca^{2+} ions also induced the folding of the RTX parallel *β*-roll motif repeat structure and played a crucial role in the AMS8 lipase structure.

5.2 Recommendation for future research

Based on the current study, the RTX parallel *β*-roll motif repeat the structure involved in the stabilization of the AMS8 lipase structure. To further explore the specific contribution of Ca^{2+} ion towards the stabilization of RTX parallel *β*-roll motif repeat structure, studies on site-directed mutation on Ca4 and Ca5 by replacement of amino acid Asp^{387} and Asp^{396} respectively to Ala could be done. Advance biophysical approach such as Small-angle X-ray scattering (SAXS) is recommended to further analyze the AMS8 lipase predicted structure since the AMS8 lipase crystal structure is not available yet.

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LIST OF PUBLICATIONS

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