UNIVERSITI PUTRA MALAYSIA

PRESSURE-INDUCED UNFOLDING OF L1 LIPASE USING MOLECULAR DYNAMICS SIMULATIONS AND QUANTUM MECHANICS CALCULATIONS

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By

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The L1 lipase derived from *Bacillus stearothermophilus* is one of the most applied enzymes that shows thermostability at 333-338K and pH 8-10. It has a tetrahedral zinc-binding site, which consists of Asp61, Asp238 from the core domain and His81, His87 from the extra domain of enzyme. L1 lipase is widely used as flavouring agent and aroma constituent in food industry due to its high substrate solubility and increased rate of reactions in the hydrolysis of fats and oils.

Major works from X-ray crystallography and nuclear magnetic resonance (NMR) experiments have successfully deciphered the structures of known enzymes, however their dynamics and flexibility foundations are still unclear. The use of pressure can monitor protein structure and its functionality in slower kinetics, compared to temperature. Most importantly, pressure is used in food processing industries to retain vitamin and nutritional contents, and reduce the viability of microorganisms.

Molecular dynamics (MD) simulations provide complementary data and valuable information to study the behaviour of macromolecules which are mostly inaccessible to experiments. From a comprehensive literature review, there is a lack of understanding on how thermoalkalophilic enzymes can unfold at high pressure, therefore 1 μs MD simulations were carried out at room temperature, to investigate the effects of 10,000 bar pressure on the structure, dynamics, and flexibility of L1 lipase. Quantum mechanics calculations were also performed by using ONIOM layer optimization to estimate the effect of high pressure on the zinc-binding site.

Based on the root-mean-square deviation (RMSD) variance at 10,000 bar, small structural changes were detected. An "unfolding-up-on-squeezing" phenomenon was clearly found as the radius of gyration (R_g) was increasing gradually despite the high compression. Our solvent accessible surface area (SASA) results also illustrated the weakening of hydrophobic forces as the pressure increased. The exposure of apolar residues to water molecules allowed the greater distribution of hydrogen bonds

between lipase and water molecules. In addition, the high desolvation energy correlated well with the changes in SASA values.

Root-mean-square fluctuation (RMSF) analysis showed that residues 75-93, 129-145 and 283-313 were highly mobile under high pressure. The flexibility at residues 75-93 was linked to the loss of tetrahedral coordination at the zinc-binding site where His81 and His87 were involved. In terms of the secondary structures, many helix-turn transitions were observed after 400 ns of simulation. Random coil was dominant at residues 265-320. There was also an indication of beta-aggregation as the beta sheets were affected by high pressure in a lesser extent, compared to helices.

Based on QM analysis, interaction at the zinc-binding site of L1 lipase was unfavourable at 10,000 bar. A lower entropy and higher enthalpy of the model system were detected. The orbital occupancy of $2p_z$ orbital of N in His81 was decreased after bound to Zn^{2+} ion at high pressure. The dipole moments were also weaker for Asp61, His81 and His87.

Overall, a complete unfolding of L1 lipase was not observed at 10,000 bar at 1 μs, but the obtained results revealed the formation of molten globule. This structure is possibly the universal folding intermediate because it is loosely packed and its structural features slightly resemble the native state of a folded protein. Therefore, it is very important in folding/unfolding pathway of enzyme.

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

PEMBUKAAN LIPATAN LIPASE L1 YANG DIPENGARUHI OLEH TEKANAN DENGAN SIMULASI DINAMIKA MOLEKUL DAN TEORI MEKANIKA KUANTUM

Oleh

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Lipase L1 yang berasal dari *Bacillus stearothermophilus* menunjukkan kestabilan yang tinggi pada 333-338K dan pH 8-10. Enzim ini mempunyai sebuah tapak pengikat zink yang berbentuk tetrahedron, meliputi Asp61, Asp 238 daripada teras utama dan His81, His87 daripada tambahan enzim yang utama. Disebabkan oleh kelarutan substrat yang baik and kadar tindak balasnya yang tinggi dalam proses hidrolisis lemak dan minyak, lipase L1 banyak digunakan sebagai bahan perisa dan aroma dalam industri pemakanan.

Hasil daripada sinar-X kristalografi dan resonans magnet nukleus (NMR) telah berjaya menafsirkan struktur-struktur enzim, namun pengetahuan asas mengenai dinamik dan fleksibiliti protein masih tidak jelas. Penggunaan tekanan boleh mengawasi struktur protein dan fungsinya menyebabkan tenaga kinetik menjadi perlahan. Di samping mengurangkan kebolehidupan mikroorganisma, industri pemprosesan makanan banyak menggunakan tekanan tinggi untuk mengekalkan kandungan vitamin dan nutrien.

Penyelidikan protein boleh dijalankan dengan simulasi dinamik molekul (MD) yang melengkapi data eksperimen. Sorotan daripada kajian menunjukkan bahawa pemahaman terhadap pembukaan lipatan enzim termoalkalofilik dalam keadaan tekanan yang tinggi masih tidak mencukupi. Oleh itu, simulasi MD dijalankan selama 1 μs, untuk menyiasat kesan 10,000 bar terhadap struktur, dinamik dan fleksibiliti lipase L1. Pengiraan melalui teori mekanik kuantum turut dijalankan dengan kaedah pengoptimuman lapisan ONIOM untuk mengkaji interaksi yang berlaku di tapak pengikat zink dalam tekanan tinggi.

Berdasarkan varians sisihan punca min kuasa dua (RMSD) pada 10,000 bar, enzim tidak membuka lipatan secara menyeluruh, namun beberapa ciri-ciri penting boleh dikenal pasti. Jejari legaran lipase L1 bertambah secara beransur dan setempat, walaupun strukturnya dimampat tekanan yang tinggi. Keluasan permukaan protein yang dimasuki oleh pelarut (SASA) menunjukkan daya hidrofobik yang semakin lemah apabila tekanan bertambah tinggi. Dedahan residu tidak polar kepada molekul air dalam tekanan tinggi juga menggalakkan ikatan hidrogen terjadi lebih banyak antara

lipase dan air. Di samping itu, tenaga disolvasi dalam lipase adalah berkaitan dengan perubahan nilai SASA dalam simulasi.

Analisis fluktuasi punca min kuasa dua (RMSF) menunjukkan bahawa residu 75-93, 129-145 dan 283-313 mempunyai fleksibiliti yang tinggi pada 10,000 bar. Fleksibiliti His81 and His87 telah menyebabkan kehilangan koordinasi tetrahedron di tapak pengikat zink dalam tekanan tinggi. Dari segi struktur sekunder, banyak heliks digantikan oleh lekukan selepas 400 ns. Gegelung rawak juga banyak kelihatan di residu 265-320. Indikasi pengagregatan kepingan beta dapat dikesan kerana bilangan strukturnya lebih konsisten sepanjang simulasi, berbanding dengan heliks alfa.

Pengoptimuman menerusi teori mekanika kuantum menunjukkan bahawa interaksi yang berlaku di tapak pengikat zink dalam 10,000 bar kurang memuaskan. Sistem tersebut melaporkan entropi yang rendah serta entalpi yang tinggi. Penghunian elektron dalam orbital $2p_z$ oleh *N* di residue His81 menjadi kurang selepas mengikat dengan Zn^{2+} ion. Momen dwikutub di Asp61, His81 dan His87 turut dikurangkan.

Secara keseluruhan, pembukaan lipatan lipase L1 tidak ditemui pada 10,000 bar selepas 1 μs, namun ciri-ciri struktur yang ditunjukkan boleh menyifatkan formasi globul lebur yang sangat penting dalam laluan perlipatan/pembukaan lipatan enzim.

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This thesis was submitted to the Senate of Universiti Malaysia and has been accepted as fulfillment 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

GROMACS	Groningen Machine for Chemical Simulations
MD	Molecular dynamics
NVT	Constant number of particles, pressure and temperature
NPT	Constant number of particles, volume, and temperature
QM	Quantum mechanics
RMSD	Root-mean-square deviation
RMSF	Root-mean-square fluctuation
SASA	Solvent accessible surface area
Ala	Alanine
Arg	Arginine
Asp	Aspartate
His	Histidine
Gly	Glycine
Ser	Serine
Tyr	Tyrosine
Zn	Zinc

CHAPTER 1

INTRODUCTION

Enzyme is a biological catalyst with the ability to increase the rate of chemical reactions. Each enzyme possesses a selective binding pocket to complement with a specific ligand. Its reactions can vary with temperature, pH and many other factors. For example, an enzyme loses its function when it is exposed to high temperature, due to the irreversible structural changes induced by heat and the failure of substrate to bind the distorted active site (Daniel and Danson, 2013).

There are enzymes which are resistant to extreme heat, known as thermoenzymes. They are originated from bacterial sources, or thermophiles that survive at approximate 333K-353K (Constantino *et al.*, 1990; Ozer and Akdemir-Evrendilek, 2014). Compared to mesozymes, they have identical catalytic mechanisms and 35-85% sequence similarity, however thermoenzymes carry additional salt bridges (Matsumura, 1989; Morgan, 2005), hydrogen bond networks, and stronger hydrophobic interactions (Vieille and Zeikus, 1996). The hydrophobic cores of thermoenzymes are usually more dense to avoid exposure to water molecules. Their loop structures are also less abundant and shorter (Thompson and Eisenberg, 1999). These characteristics reduce the entropy of unfolding at high temperature.

Thermoalkalophilic enzymes, especially lipases have the ability to adapt alkaline environment (pH 8.0–11.0) (George-Okafor and Odibo, 2011). They are also highly stable in organic solvents such as propanol, acetone and methanol (Schmidt-Dannert, 1994). Biocatalysts derived from *Achromobacter*, *Arthrobacter*, *Bacillus*, and *Pseudomonas* (Gupta *et al.*, 2004) do not require cofactors in any biological activities (Gupta *et al.*, 2004; Treichel *et al.*, 2010). These enzymes are highly sought after in industries nowadays, with widespread applications in detergent, pharmaceuticals and biodiesel productions (Balan *et al.*, 2013).

L1 lipase is a thermoalkalophilc enzyme grouped under lipase family I.5 (Kim *et al.*, 1998; Kim *et al.*, 2000). This lipase is thermoactive at 333-338K and at alkaline pH 9-10 (Kim *et al.*, 1998). A thermoactivity test using olive oil emulsion also revealed the optimum activity of lipase up to 341K when tested at pH 8, in the absence of detergents (Kim *et al.*, 2000).

Industries show a lot of interest in this enzyme due to its resistance to denaturing agents, for example, proteases, detergents, and organic solvents (Eijsink *et al.*, 1992). The use of L1 lipase in chemical and biochemical reactions increases the hydrolysis rates of fats and oils with high contents of saturated fatty acids, for example, palm oil, coconut oil and cotton seed oil (Kim *et al.*, 2000). It also shows substrate specificity towards tripropionin and p-nitrophenyl caprylate (Kim *et al.*, 1998).

To highlight the importance of thermostability in enzymes, characterization studies on tightly folded protein conformations or polypeptide chains are encouraged. Many experiments have routine use of denaturants in biochemical laboratory such as heat, extreme pH, detergents, urea and guanidinium chloride to induce protein unfolding and

alter its solvent environment (Bennion and Daggett, 2003; Konermann, 2012; Jacso *et al.*, 2013).

However, folded proteins are marginally stable (Taverna and Goldstein, 2002). Subtle changes in the physical and chemical properties of solvent can shift the folding equilibrium easily, therefore it is difficult to observe the transitions states for protein folding/unfolding. As of today, the pressure-induced unfolding of enzyme is still elusive. The role of pressure in protein stability should not be disregarded because it is very effective. Vitamins and nutritional contents in food are preserved in specific conditions (Oey *et al.*, 2008). Thus, there is a possibility to modify the enzymatic nature of protein and increase food digestibility using pressure (Zhang *et al.*, 2011; Tokusoglu *et al.*, 2014). Pressure also has the potential to suppress the growth of microorganisms and the viability of viral particles in biochemical processes (Jaenicke *et al.*, 1981; Sharma *et al.*, 2002; Ishii *et al.*, 2004; Gaspar *et al.*, 2008). Other applications in pharmacology and drug design using high pressure have also been reported. For example in Alzheimer's disease, high pressure has the tendency to slow down the formation of amyloid-ß-peptide aggregates and modulate the dissolution of preformed fibrils (Silva *et al.*, 1993; Dobson *et al.*, 2003)

Functional proteins are large and complex, compared to the oligomeric proteins and fast-folding peptides. The pressures of 3,000 bar and above are required to unfold globular proteins (Doster and Gebhardt, 2003), while pressures beyond 10,000 bar are needed for bacterial proteins, according to Sharma (2002). Since experimental methods cover the core of scientific researches, Pandharipande and Makhatadze (2015) used pressure perturbation calorimetry to observe the volume changes in ubiquitin, acylphosphatase (ACP) and tryptophan zipper (TrpZip) as they became destabilized at high pressure. High-pressure spectroscopy and site-directed spin labelling EPR (SDSL-EPR) were also used to detect structural changes in the different transition states of myoglobin at 0-2,400 bar (Lerch *et al.*, 2013).

However, the details that underlie the pressure-induced unfolding process of protein are still unclear from experimental procedures. Time-dependent events such as the secondary structure transitions, residue interactions, and changes in structural compactness may be neglected (Pfeil and Privalov, 1976; Makhatadze and Privalov, 1992; Myers and Oas, 2002; Lin *et al.*, 2011). In addition, denaturing agents can associate irreversibly with the unfolded states of protein to form aggregates and undergo proteolysis, which will hinder the characterization of enzyme (Paliwal *et al.*, 2004). Experimental measurements are difficult to be carried out in biological environments as scientists need to overcome practical limitations like spatial and temporal resolutions to access the orbital energies, occupancies and bonding information (Gerstman and Prem, 2009; Xu *et al.*, 2013), unless ultrafast electron diffraction crystallography and microscopy methods are applied (Thomas and Zewail, 2008; Shorokhov and Zewail, 2008).

In recent decades, computational methods are introduced profoundly to help rationalize problems arised from various disciplines, including chemistry, physics, mathematics and biology (Lipkowitz and Boyd, 1998; He, 2000; Zoete and Meuwly, 2006; Benamou *et al.*, 2014). The increasing accuracy and the high-speed calculations of complex systems using computers have attracted many scientists to combine *in silico* methods and wet lab experiments (Pulay, 2011). Data obtained from lab-based studies can be validated theoretically to explain the geometry and the physical properties of the

target molecules (Tysoe, 2013; Nasica-Labouze *et al.*, 2015). Atomic details which are inaccessible to experiments can be found by computational calculations (Ng, 2015) such as Monte Carlo (MC), molecular dynamics (MD), molecular docking and quantum mechanics calculations.
For example, Frappier and Najmanovich (2014) demonstrated the effects of mutation on thermostability using the normal mode analysis of an elastic network atomic model. Additonally, Zhou and Grigoryan (2015) utilized the Protein Data Bank library to study specific tertiary structural elements which could contribute towards alterations in protein structures.

The use of molecular dynamics (MD) simulation allows scientists to understand how particles move in a given space as a function of time (Karplus and Kuriyan, 2005). From the Newton's second law of motion, the force applied to any system is equivalent to the mass and the acceleration of moving particles, thus the trajectory produced will contain information related to the positions, the velocities and the accelerations of particles in the system under study (Vikramraja JS, 2008). MD allows researchers to map non-equilibrium events during the denaturation of protein (Beck and Daggett, 2004), in all-atom or united atom internal representations (Zheng and Glenn, 2015). It assesses the transitions of macromolecules from their native forms towards the intermediates and unfolded states (Huynh *et al.*, 2002).

High temperature is commonly used in MD simulation to induce the folding/unfolding of protein structures (Karplus and Sali, 1995; Day *et al.*, 2002; Settani and Fersht, 2007; Rocco *et al.*, 2008), however the risk of irreversible aggregation is high for thermally-denatured proteins (Heremans and Smeller, 1998).

The application of pressure in MD related studies can help us understand the change in intrinsic compressibility and the volumetric properties of a protein (Paci and Marchi, 1996), which is also a good indication of protein unfolding. Proteins under compression may have slower folding/unfolding processes, but pressure can destabilize hydrophobic contacts in proteins (Grigera and McCarthy 2010). water penetration models have been suggested to improve their kinetics (Weber and Drickamer, 1983; Hummer *et al.*, 1998; Imai and Sugita, 2010; Sarupria *et al.*, 2010).

1.1 Objectives

The main objective of this research was to investigate the stability and the unfolding pathway of L1 lipase under extreme pressure using MD simulations and quantum mechanics calculations. Therefore, these specific objectives were explored:

- To model L1 lipase unfolding at high pressure.
- To determine the dynamics and the flexibility of L1 lipase at high pressure.
- To identify the structural changes of L1 lipase at high pressure during unfolding.
- To estimate the stability of enzyme at 10,000 bar by focussing on protein zinc-binding site.

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