

## DESIGN OF *CANDIDA ANTARCTICA* LIPASE B THERMOSTABILITY IMPROVEMENT BY INTRODUCING EXTRA DISULFIDE BOND INTO THE ENZYME

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### ABSTRACT

*Candida Antarctica* Lipase B (CALB) is extensively studied in enzymatic production of biodiesel, pharmaceutical products, detergents and other chemicals. One drawback of using CALB is its relatively low optimum temperature at 313 K (40°C). The objective of this research is to design CALB mutant with improved thermostability by introducing extra disulfide bond. Molecular dynamic simulation was conducted to get better insight into the process of thermal denaturation or unfolding in CALB. Thermal denaturation of CALB was accelerated by conducting simulation at high temperature. Molecular dynamic simulation of CALB was performed with GROMACS software package at 300-700 K. Prediction of possible mutation was done using "Disulfide by Design<sup>TM</sup>" software. Selection of mutated residues was based on flexibility analysis of CALB. From those analyses, three mutants were designed, which are Mutant-1 (73LeuCys/151AlaCys), Mutant-2 (155TrpCys/294GluCys) and Mutant-3 (43ThrCys/67SerCys). Parameters that were used to compare the thermostability of mutant with wild type enzyme were Root Mean Square Deviations (RMSD), Solvent Accessible Surface Area (SASA), Radius of gyration (Rg) and secondary structure. Molecular dynamic simulation conducted on those three mutants showed that Mutant-1 has better thermostability compared to wild type CALB. We proposed the order of mutant thermostability improvement as follows: Mutant-1, Mutant-2 and Mutant-3, with Mutant-1 having better potential thermostability improvement and Mutant-3, the least stable.

**Keywords:** *Candida antarctica* Lipase B, Thermostability, Molecular Dynamic Simulation, Mutation, Disulfide Bond

### 1. INTRODUCTION

Lipase is a group of enzyme, which members are utilized in many fields of application such as production process of detergent, surfactant, food, pharmaceutical product, cosmetic, paper and pulp and nutrition (Sharma *et al.*, 2001; Bisht *et al.*, 2012). According to Ganjalikhany *et al.* (2012), Lipase B of *Candida antarctica* (*C. antarctica* lipase B, CALB) is extensively studied in catalyzing chemical reaction such as resolution of racemic compounds (Patel *et al.*, 2000), biodiesel production (Zhao *et al.*, 2007) and ring-

opening polymerization (Peeters *et al.*, 2005). CALB is highly enantioselective, can utilize wide range of substrate and has a good stability in organic medium (Ong *et al.*, 2006). However, the drawback of using CALB is its low thermostability, where the optimum working temperature of CALB is at 40°C and its stability and activity decrease at higher temperature (Mittelbach, 1990; Xu *et al.*, 2003; Suen *et al.*, 2004; Chodorge *et al.*, 2005; Ong *et al.*, 2006). Enzyme with high thermostability is more favored because many industrial processes require higher temperature to increase the reaction rate, decrease substrate solubility

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and reduce contamination (Haki and Rakshit, 2003; Ong *et al.*, 2006).

Mutation of protein via genetic engineering to change its amino acid sequences is one strategy that can be utilized to improve certain protein properties such as thermostability (Subramanian and Natarajan, 2011). One mutational strategy to engineer a protein in order to improve its thermostability is by introducing new/extra disulfide bond into the protein molecule (Ling *et al.*, 2009). This is conducted by mutating two (a pair) amino acid residues to cysteine (Tambunan *et al.*, 2011b). Successful examples of thermostability improvement by adding new disulfide bond were phage T4 lysozyme (Matsumura *et al.*, 1989) and *Bacillus circulans* xylanase (Wakarchuk *et al.*, 1994). Disulfide bond is known to stabilize protein both enthalpically and entropically. Decreasing denatured state entropy can be considered as the major effect of introducing disulfide bond, thus favouring the native state (Betz, 1993; Yamaguchi *et al.*, 1996; Babakhouya *et al.*, 2010), although enthalpic stabilization of native state should also be considered (Betz, 1993). Analyzing flexible area in protein that shows high movement can be utilized to determine potential mutation target. This highly flexible area is considered to play a role in early unfolding process and improving rigidity of this area, e.g., introducing disulfide bond can potentially quench unfolding process (Liu and Wang, 2003).

Recent developments in computational biology such as molecular dynamic simulation and molecular modeling can help the task on engineering certain protein properties (Vijayan *et al.*, 2007). Computer aided design has become a common practice now in protein engineering, especially as an initial step to design a mutant enzyme and to simulate certain properties of the mutant (Yamaguchi *et al.*, 1996; Santarossa *et al.*, 2005; Sandström *et al.*, 2009; Han *et al.*, 2009; Kim *et al.*, 2010). Molecular dynamic simulation helps researcher to study the physical changes in protein in their response to temperature changes (Li and Daggett, 1994; Day *et al.*, 2002; Liu and Wang, 2003; Purmonen *et al.*, 2007; Paschek *et al.*, 2011). Analysis of flexible area in protein during temperature changes can be performed by molecular dynamic simulation.

The objective of this study is to design CALB mutant that has improved thermostability by introducing extra disulfide bond. Molecular dynamic simulation was utilized to analyze protein structural changes during elevated temperature and analyze the thermostability of mutants represented by several physical properties (Harishchander *et al.*, 2010). Thermal denaturation of

CALB was accelerated by conducting simulation at high temperature. Obtained mutant design could be proposed to be implemented for site-directed mutagenesis of CALB.

## 2. MATERIALS AND METHODS

### 2.1. Molecular Dynamic Simulation

Molecular dynamic simulation was conducted with GROMACS package (Van Der Spoel *et al.*, 2005; Hess *et al.*, 2008) running in Ubuntu 10.04 LTS. Model visualization and analysis was conducted with GROMACS package and VMD 1.9 (Humphrey *et al.*, 1996). CALB crystal structure was PDB 1TCA (Uppenberg *et al.*, 1994) with every water molecule and N-acetyl-D-glucosamine in the crystal removed prior to simulation. AMBER99 (Wang *et al.*, 2000) force field was used to define potential energy of the model. Solvation of the system was conducted using TIP3P water molecule (Jorgensen *et al.*, 1983). Wild type CALB simulation system was prepared in a dodecahedron box with total volume of 350.77 nm<sup>3</sup>. Sodium ion was added to neutralize the total charge. This whole system consisted of 1 CALB molecule, 10284 TIP3P water molecule and one sodium ion.

### 2.2. System Minimization

System minimization was conducted in two steps: 1000 steps of steepest descent, followed by 1000 steps of conjugate gradient (Payne *et al.*, 2013). Minimization was followed by short simulation for 10 ps with positional restraint on protein molecules. NVT ensemble was used in simulation by coupling the system to velocity rescaling thermostat using coupling constant of 0.1 (Bussi *et al.*, 2007; Larsson *et al.*, 2012). Electrostatic and van der Waals interactions were modeled with Particle Mesh Ewald (PME) (Essman *et al.*, 1995) and Lennard-Jones potential, respectively. Each was calculated in 1.0 nm cut-off. LINCS algorithm (Hess *et al.*, 1997) was used to constrain every bond that involves hydrogen atom.

Main simulation steps were heating to desired temperature for 10 ps, followed by 100 ps equilibration and production for 6000 ps. Average computational speed was around 2.3 ns/day; thus 6000 ps simulation time would take about 3 days of real computing time.

### 2.3. Mutation of the Amino Acids

Candidate of amino acid residues that can be mutated to cysteine were created by Disulfide by Design<sup>TM</sup> software (Benson and Daggett, 2008). The crystal

structure of CALB after minimization step was used as an input for this software. Parameters for creating mutation candidate were Optimum *Chi3-angle* = +100°/-80°, *tolerance* ±10°; and *Ca-Cb-S-angle* = 114.60°, *tolerance* ±10°. Model of CALB mutant molecules was also generated using this software by selecting desired pair of amino acids. Mutant models were prepared in same simulation system as the wild type CALB. Sodium or chloride ion was added when necessary as counter ion to neutralize the total charge of simulation system (Senn and Thiel, 2009). Simulation of mutants CALB was also done using the same minimization steps and simulation parameters.

### 3. RESULTS

#### 3.1. Validation of Model

Validation of a simulation system or model can be done by comparing simulation data with experimental data (Chandramat *et al.*, 2005; Lim *et al.*, 2010). Some simulation data that can be used are density, salivation free energy, viscosity, surface tension and crystallographic B-factor (Pikkemaat *et al.*, 2002). In this study, B-factor from experimental data of PDB 1TCA was used and compared with B-factor from simulation. We used C- $\alpha$  atoms RMSF from simulation of wild type CALB at 300 K, specifically from 4000-6000 ps. Bfactors values were determined from RMSF value using the following equation (Kuzmanic and Zagrovic, 2010):

$$\text{RMSF}_i^2 = \frac{3B_i}{8\pi^2}$$

**Figure 1** shows crystallographic B-factor and simulation B-factor. Overall observation shows that most crystallographic and simulation B-factor values do not differ significantly and both shows similar pattern. Large difference was observed for residues 4-9, 12-20, 141-150 and 285-289. This particularly resulted from the different conditions of B-factor obtained. Simulation was performed on a solvated model, where protein molecules got more flexible and more protein movement was expected compared to more rigid crystal state. Overall, we conclude that this model has good validity to be able to represent desired experiment.

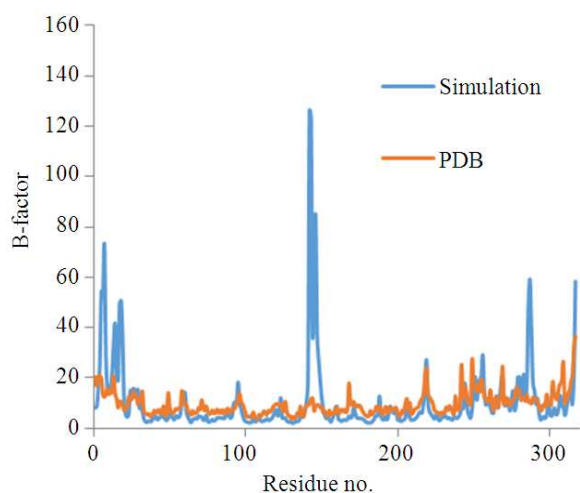
#### 3.2. Mutant Selection

Mutant selection was based on flexibility analysis by considering RMSF analysis and mutant prediction by Disulfide by Design<sup>TM</sup> software (Subramanian and

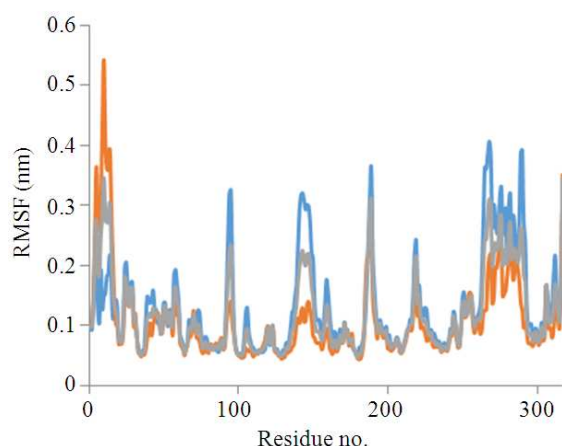
Natarajan, 2011). First, we determined highly flexible residue in CALB as mutation candidate. Highly flexible residues were generally considered to play a role in early unfolding process. Introducing more rigid property, like building a disulfide bond, would limit the movement in this flexible area and help in quenching the transition to unfolded state (Pikkemaat *et al.*, 2002; Liu and Wang, 2003; Jeong *et al.*, 2007; Han *et al.*, 2009). Here, we use RMSF value to represent atomic movement in the backbone atoms, especially C- $\alpha$  atoms in varied simulation temperatures.

Simulation on varied temperature shows that higher temperature gave larger RMSF value (**Fig. 2**). We conducted simulations up to 700 K, but here we only used RMSF value up to 400 K. We set a minimal RMSF value of 0.05 nm as a criterion where CALB is still considered in the native state (Benson and Daggett, 2008). We exclude the RMSF data of 450-700 K simulation because we consider that CALB molecules in these simulations were already in the unfolded state (all RMSF values >0.5) and include RMSF data of 300-400 K because they still have some residues with RMSF value of ≤0. There are nine residue areas that have high flexibility: Residues 1-30, 43-62, 91-97, 140-153, 168-173, 184-203, 210-225, 238-297 and 304-317.

RMSF value was taken from simulation at 4000-6000 ns of each temperature. Disulfide by Design<sup>TM</sup> was used to generate pair of residues that are likely to form disulfide bond when mutated to cysteine based on geometric properties of disulfide bond such as C $\beta$ -S $\gamma$  and S $\gamma$ -S $\gamma$  bond length and C $\beta$ -S $\gamma$ -S $\gamma$  angle (Dombkowski, 2003). The software predicted twelve pairs of residues in CALB that could be mutated to cysteine and form disulfide bond (**Table 1**). On the other hand, it can also analyze three disulfide bonds that already exist in CALB, which prove good validity of the software. There were no residues suggested that involved in catalytic, binding or oxyanion hole sites. Results from flexibility analysis and software prediction were combined to generate CALB mutants. We exclude residues that are assigned in the middle of helix or strand structure because adding disulfide bond to these structure would add extra restrain on the structure (Pikkemaat *et al.*, 2002). Matching these three criteria (flexibility, not assigned in secondary structure and software output), we obtained three pairs of potential mutation to cysteine, which are: 73Leu-151Ala, 155Trp-294Glu and 43Thr-67Ser. Three mutant models were generated using Disulfide by Design<sup>TM</sup> from those mutations: Mutant-1 (Leu73Cys/Ala151Cys), Mutant-2 (Trp155Cys/Glu294Cys) and Mutant-3 (Thr43Cys/Ser67Cys).



**Fig.1.** Crystallographic B-factor experiment (PDB 1TCA) compared to simulation B-factor



**Fig. 2.** RMSF analysis of CALB simulation 300 K (orange), 350 K (grey), and 400 K (blue)

**Table 1.** Software prediction (disulfide by Design<sup>TM</sup>) of pair of residues that can likely form disulfide bond when mutated to cysteine

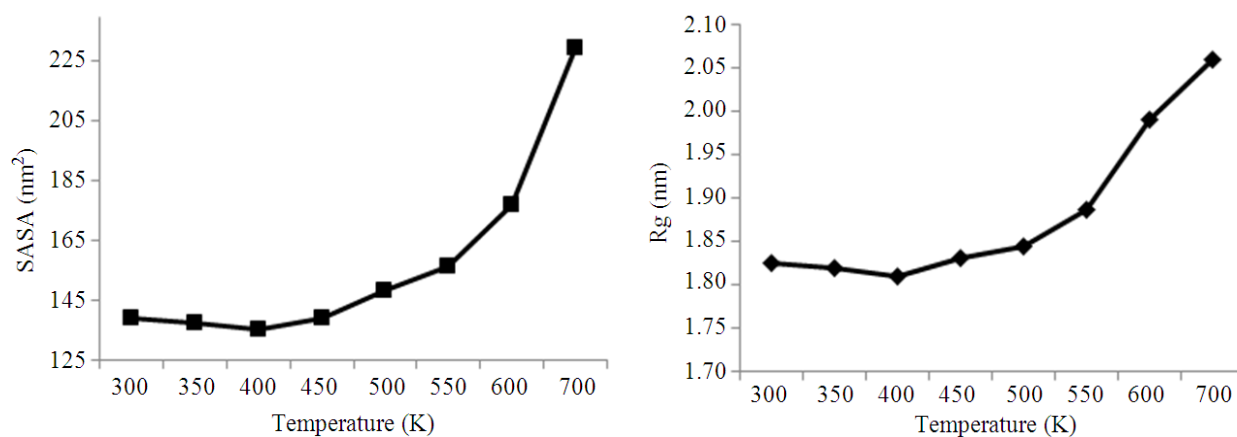
Parameter
42Thr-46Gln
43Thr-67Ser
50Ser-274Ala
73Leu-151Ala
155Trp-94Glu
167Leu-72Gly
183Tyr-202Ser
208Lys-47Gln
234Tyr-63Ala
239Ser-52Asp
266Leu-70Gln

### 3.3. Molecular Dynamics Simulation of Mutants

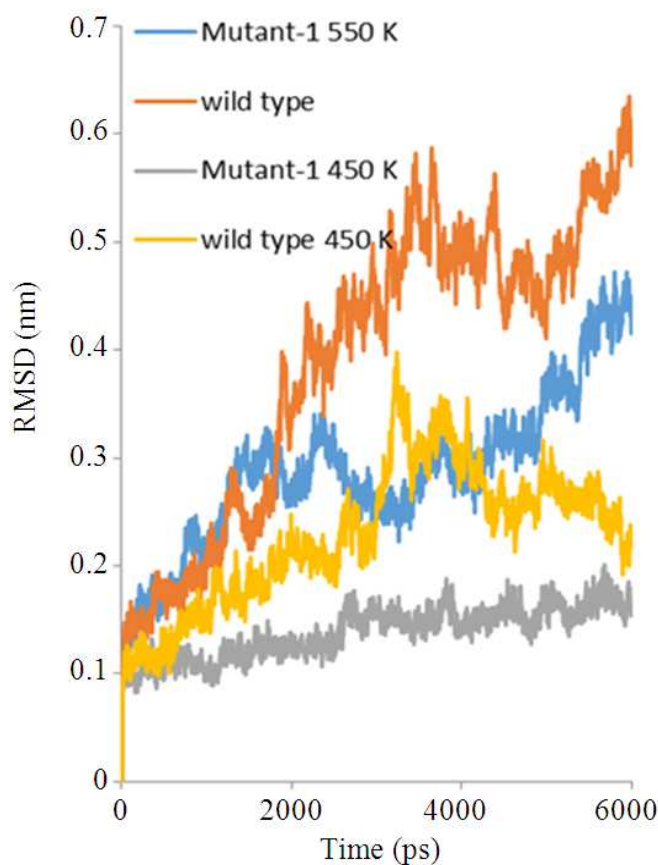
All three mutants were simulated at 450 and 550 K simulation temperature. These two temperatures were chosen because at 450 K, the physical parameters (radius of gyration and solvent accessible surface area) of wild type CALB just started to give significant increase, while at 550 K rapid structural changes were observed (**Fig. 3**) (Ceruso *et al.*, 2004). Thus, 450 K simulation represents the start of unfolding process and 550 K simulation represents the unfolded state. We compare the physical parameter (RMSD, SASA and Rg) of wild type and mutants to represent the thermostability of both molecules (Gundampati *et al.*, 2013). Significant increase in Solvent Accessible Surface Area (SASA) will happen when a protein molecule starts to lose its stabilizing interactions (intermolecular hydrogen bond, electrostatic and van der Waals interaction) and hydrophobic core of protein molecule starts to collapse; thus the protein will be more readily accessible to the solvent molecule (Day *et al.*, 2002; Yan *et al.*, 2010). According to this explanation, more linear SASA value shows more stability in the protein structure along simulation time. Radius of gyration (Rg) can also be used to relate protein stability in terms of unfolding process (Li and Daggett, 1994; Day *et al.*, 2002; Zhao *et al.*, 2009; Paschek *et al.*, 2011).

RMSD analysis on Mutant-1 and wild type on 450 and 550 K simulation showed that Mutant-1 can maintain its structural stability better compared to wild type (**Fig. 4**). In both temperatures, final RMSD value of Mutant-1 is smaller than wild type. At 450 K simulation, Mutant-1 reaches linearity faster than wild type. This indicates that Mutant-1 is capable of maintaining its structural stability better than wild type because RMSD represents the structural differences in each simulation time compared to its initial structure. Investigation on SASA and Rg value also indicates the stability of Mutant-1 relative to wild type (**Fig. 5 and 6**). There was no significant difference at 450 K simulation; however, Mutant-1 maintains its structural stability better at 550 K. This is shown by the ability of Mutant-1 to maintain the linear trend of RMSD longer. At 550 K, wild type CALB started to give a jump in RMSD value at 1000 ps, while Mutant-1 still gave linear RMSD up to 4000 ps.

Mutant-2 also shows relative stability to wild type for RMSD value at 450 K simulation; meanwhile at 550 K the RMSD of both did not differ significantly (**Fig. 7**).

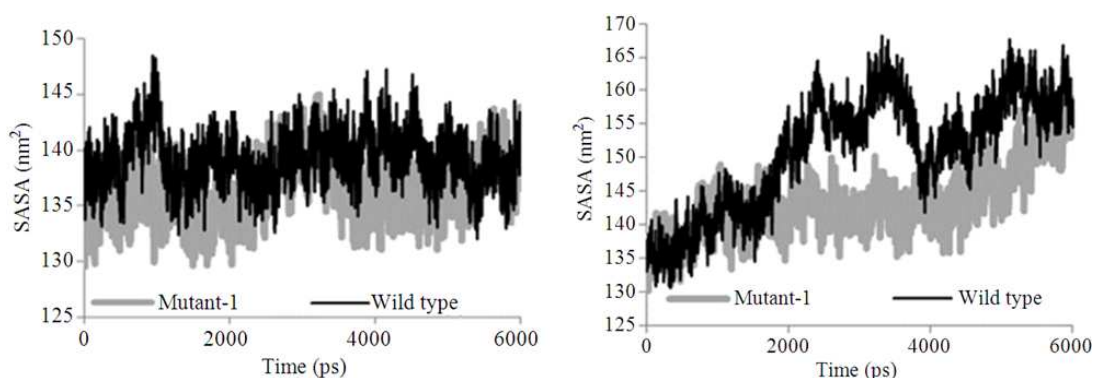


**Fig.3.** Average solvent accessible surface area. (Up) Average Solvent Accessible Surface Area (SASA) of final 2000 ps simulation of wild type CALB; and (Down) average Radius of gyration (Rg) of final 2000 ps simulation of wild type CALB

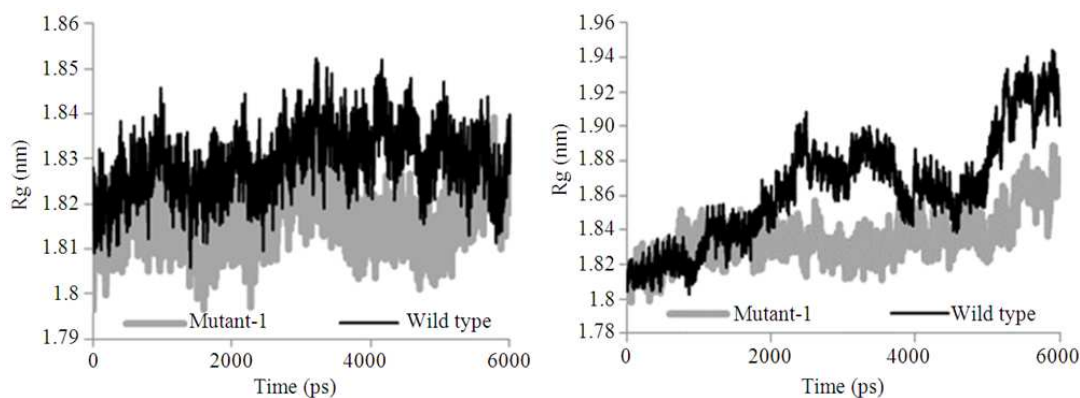


**Fig. 4.** RMSD value of Mutant-1 and wild type CALB at 450 and 550 K simulation. Mutant-1 at 550 K (blue), wild type CALB at 550 K (orange), Mutant-1 at 450 K (grey), wild type CALB at 450 K (yellow)

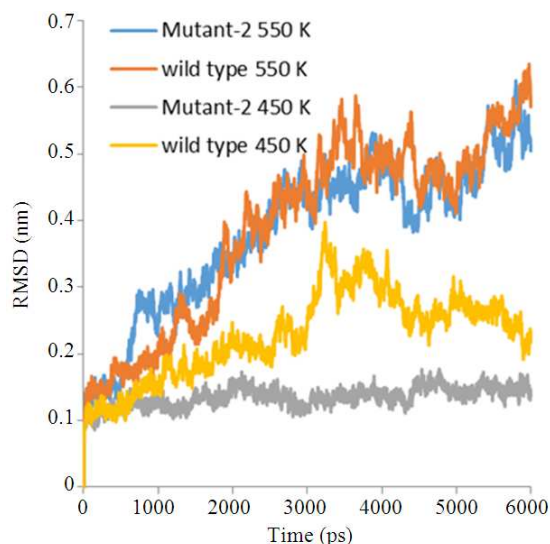




**Fig. 5.** SASA value of Mutant-1 and wild type CALB 450 K (up) and 550 K (down) simulation



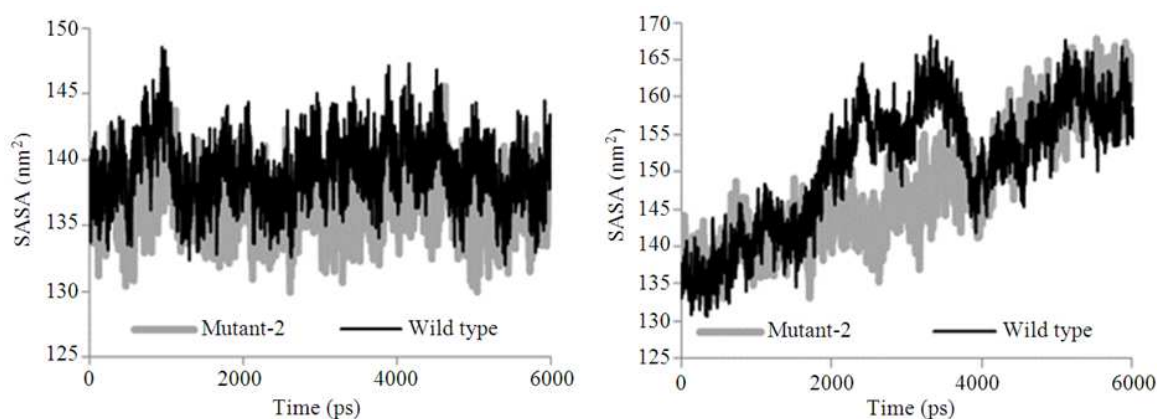
**Fig. 6.** Rg value of Mutant-1 and wild type CALB 450 K (up) and 550 K (down) simulation



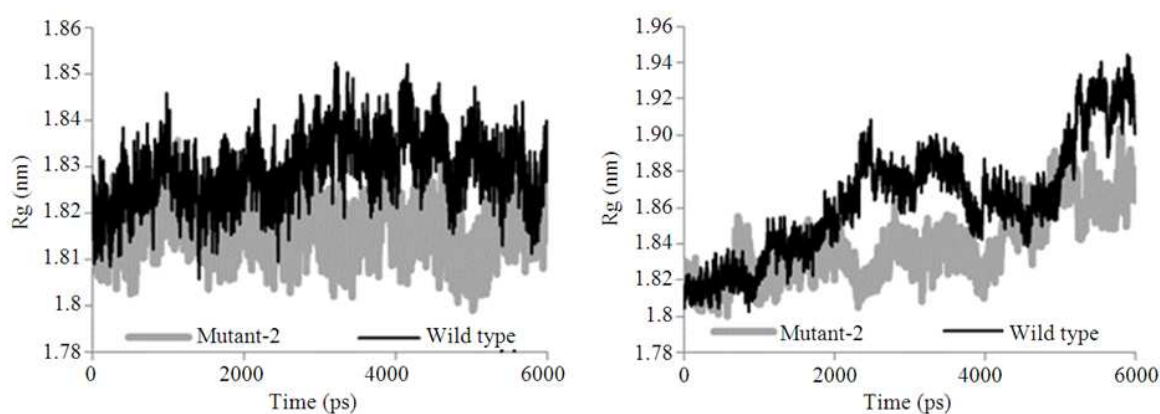
**Fig. 7.** RMSD value of Mutant-2 and wild type CALB at 450 and 550 K simulation Mutant-2 at 550 K (blue), wild type CALB at 550 K (orange), Mutant-2 at 450 K (grey), wild type CALB at 450 K (yellow)

This might suggest that Mutant-2 was more thermostable than wild type, but not as stable as Mutant-1. Further analysis on SASA and Rg also supports this conclusion. At 450 K, both SASA and Rg values of Mutant-2 and wild type show close value between them (**Fig. 8 and 9**). At 550, the final SASA and Rg values of Mutant-2 and wild type were close to each other; although along the simulations there were some period of times that Mutant-2 gave lower value of SASA and Rg. All of these analyses might support the conclusion that Mutant-2 still has better thermostability than wild type, but less stable compared to Mutant-1.

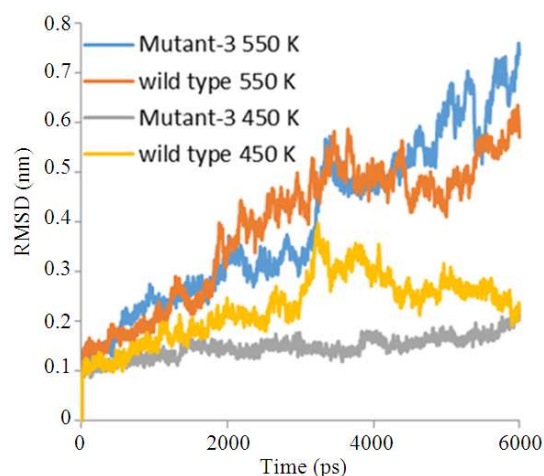
Among the three generated mutants, Mutant-3 shows the least stability. Simulation of Mutant-3 at 450 K shows that Mutant-3 indeed maintains its stability by slowly increasing RMSD value, although final RMSD values were close to each other (**Fig. 10**). At 550 K, both RMSD along simulation did not differ much; in fact, the final RMSD value of Mutant-3 exceeded the wild type. Meanwhile, the SASA and Rg value of Mutant-3 did not differ from the wild type at both simulation temperatures.



**Fig. 8.** SASA value of Mutant-2 and wild type CALB 450 K (up) and 550 K (down) simulation



**Fig. 9.** Rg value of Mutant-2 and wild type CALB 450 K (up) and 550 K (down) simulation



**Fig. 10.** RMSD value of Mutant-3 and wild type CALB at 450 and 550 K simulation Mutant-3 at 550 K (blue), wild type CALB at 550 K (orange), Mutant-3 at 450 K (grey), wild type CALB at 450 K (yellow)

## 4. DISCUSSION

Molecular dynamic simulation is an efficient method that could be utilized for constructing model on protein conformation and time repertoire (Adcock and McCammon, 2006; Ian *et al.*, 2013; Carrillo-Vázquez *et al.*, 2013). As we can see, molecular dynamic simulation is still the most effective way to model the structure and reactivity of an enzyme (Gabel *et al.*, 2009; Lousa *et al.*, 2012; Christensen and Kepp, 2013). In this respect, we have utilized this method since 2011 (Tambunan *et al.*, 2011a; 2011b; 2013; Tambunan and Parikesit, 2012; Parikesit *et al.*, 2013).

Previously, our research group has already produced result on the optimization of enzyme stability, albeit with different types of enzyme (Idrus and Tambunan, 2012). Moreover, we already have experience in conducting mutation study (Tambunan *et al.*, 2012). Hence, we utilized the existing pipeline for this research, albeit with optimization process for CALD.

Although it is not already wide spread in industrial scale, enzyme stability studies have been conducted extensively in Indonesia (Susilowati *et al.*, 2008; Nurcholis *et al.*, 2012; Hanim *et al.*, 2013). Eventually, the modeling study has been conducted as well (Hertadi *et al.*, 2007). The investment of private companies in Indonesian bio-industry is already available in segmented market (Sukara and Slamet-Loedin, 2000). To this end, we are optimistic that our developed pipeline will be utilized in local bio-industry. Henceforth, bio-industry needs this pipeline, as the one way to increase productivity is by introducing useful mutation in the enzyme.

The molecular dynamic method has successfully determined the amino acid residues with the higher tendency toward flexibility (Noorbatcha *et al.*, 2010). Thus, this modeling research has successfully sealed down the flexibility tendency, by introducing disulfidebased mutation and increasing the stability of the enzyme. To this end, by using bioinformatics tools, it is possible to design a PCR primer that could introduce the mutation by using site directed mutagenesis method (Wang *et al.*, 2009).

## 5. CONCLUSION

Thermostability analyses of mutants CALB were conducted by using physical parameters such as RMSD, solvent accessible surface area and radius of gyration. These parameters were used to represent structural changes in the process of protein unfolding influenced by temperature. From three mutants generated, the physical parameters showed that all of them might have better thermostability compared to wild type. We proposed the order of mutant thermostability improvement as follows: Mutant-1, Mutant-2 and Mutant-3; with Mutant-1 potentially having better thermostability improvement and Mutant-3, the least stable. This study only predicts the thermostability criteria based on physical properties. Other analysis could be conducted in modeling thermostability improvement; among them is free energy difference between mutant and wild type ( $\Delta\Delta G$ ).

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