

## Rational protein design for enhancing thermal stability of industrial enzymes

Le Quang Anh Tuan<sup>1\*</sup>

<sup>1</sup>Ho Chi Minh City Open University, Vietnam

\*Corresponding author: tuan.lqa@ou.edu.vn

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### ABSTRACT

Enzymes possessing many excellent properties such as high selectivity, consuming less energy, and producing fewer side products or waste have been widely applied as biocatalysts in pharmaceutical production and many industries such as biofuel, biomaterials, biosensor, food, and environmental treatment. Although enzymes have shown their potential as biocatalysts for many industrial applications, natural enzymes were not originated for the manufacturing process which requires harsh reaction conditions such as high temperature, alkaline pH, and organics solvents. It was reported that reduction of the final conversion of several enzymatic reactions was declined at high temperature. Protein engineering to improve the enzymes' thermostability is crucial to extend the use of the industrial enzymes and maximize the effectiveness of the enzyme-based processes. Various industrial enzymes with improved thermostability were produced through rational protein engineering using different strategies. This review is not aimed to cover all successful rational protein engineering studies. The review focuses on some effective strategies which have been widely used to increase the thermostability of several industrial enzymes through the introduction of disulfide bonds and the introduction of proline.

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### 1. Introduction

Enzymes as biocatalysts have been widely used for the synthesis of molecules for the pharmaceutical and industrial sector due to their excellent properties such as being highly selective, consuming less energy, and producing fewer side products or waste (Bornscheuer et al., 2012), (Bornscheuer & Pohl, 2001), (Lutz, 2010), (Woodley, 2006), (Akoh, Chang, Lee, & Shaw, 2007). In addition, many enzymes do not require toxic metal ions for their catalytic properties; thus, it would be beneficial for applying them in environmentally benign processing for the production of industrial products (P. Turner, Mamo, & Karlsson, 2007). Enzymes were applied as biocatalysts for various industries such as biofuel, biomaterials, biosensors, food, and environmental treatment. Lipases were used for the production of biodiesel (Ko'ise, Tu'ter, & Aksoy, 2002), (Shimadaa et al., 1999), (Akoh et al., 2007) and polysaccharide-degrading

enzymes such as cellulases and xylanases were used for production of second-generation ethanol for biofuel from cellulosic biomass (Saha, 2003), (Wang et al., 2014), (Badiéyan, Bevan, & Zhang, 2012). The applications of enzymes for the production of various industrial products are seen in Table 1. The global market for industrial enzymes reached nearly \$4.9 billion in 2015. Furthermore, the market is expected to grow 4.7% annually from 2016 through 2021 (Business Communications Company, 2017).

To apply enzymes for the production of industrial products, the thermal stability of the enzymes is one of the most desired characteristics because enzyme-catalyzed reactions increase exponentially with increasing reaction temperature up to enzyme denaturation temperature (Peterson, 2007). The Conversion of biodiesel (i.e., methyl esters) from cotton-seed oil and methanol catalyzed by immobilized *Candida antarctica* lipase B, for example, increased with increasing reaction temperature in the range of 30-50°C and reached maximum methyl ester yield of 91.5% at 50°C after 7h. In addition, the reactions at high temperatures are essential for increasing the solubility and/or decreasing viscosity of reactants as well as decreasing the risk of bacterial contamination (Eijsink et al., 2004). Production of high fructose corn syrup (HFCS), for example, the liquefaction of starch is usually performed at high temperature to increase the solubility of starch which is insoluble in cold water (e.g., the liquefaction of starch into soluble, short-chain dextrin catalyzed by  $\alpha$ -amylases is performed 95-105°C for 90 minutes (Maarel, Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002). Thermostable enzymes have many advantages in promoting better enzyme penetration and cell-wall disorganization of the raw materials in biorefinery which produces chemicals, commodities and fuels from renewable raw materials such as agricultural crops (P. Turner et al., 2007). Thermostable enzymes also provide opportunities to perform the reaction at high temperature to reduce the risk of microbial contamination such as *Lactobacillus* in the ethanol production from non-sterilized cellulosic biomass (Badiéyan et al., 2012). Conversion of starch to oligomaltodextrin in production of high fructose corn syrup catalyzed by  $\alpha$ -amylase, for instance, required the high thermal stable enzyme because the process concomitantly injected with steam (Kirk, 2002).

Although the enzymes have shown their potential as biocatalysts for many industrial applications, natural enzymes were not originated for the manufacturing process which requires harsh reaction conditions such as high temperature, alkaline pH, and organics solvents. It has been found that the reduction of the final conversion of several enzymatic reactions was declined at high temperatures. For example, the conversion of glycerol carbonate and biodiesel synthesis which are catalyzed by *Candida antarctica* lipase B were significantly decreased around 60°C (Ko'se et al., 2002); (S. Kim, Kim, Lee, Yoon, & Song, 2007). Enhancing the thermostability of the applied enzymes would be beneficial for extension and maximizing the effectiveness of the enzyme-based processes.

Protein engineering has been increasingly considered as an excellent choice to fulfill the above requirement. Protein engineering, one part of genetic engineering, performs the modification of protein sequence through mutations (i.e., single or multiple mutation at certain amino acid residues) to change the properties of a protein. Protein engineering is classified into two groups: directed evolution and rational protein design. The directed evolution (also called molecular evolution) evolves the protein characteristics through random mutagenesis of the gene encoding for target protein (e.g., error-prone PCR) or through the combination of different

fragments of DNA in the molecular pool (Bornscheuer & Pohl, 2001), (N. J. Turner, 2009). In this approach, the number of variants in the library was so huge and it requires an efficient high-throughput screening to select the evolved variants. In contrast to the directed evolution, the rational protein design evolves the enzyme characteristics through site-directed mutagenesis at one or several certain amino acid residues which were predicted by analysis of sequence, structure, function of target enzymes (Bornscheuer & Pohl, 2001). In the rational protein design, the number of variants in experiments is relatively small and results depend highly on the prediction step. However, knowledge and information on the sequence and/or structure of the target enzyme is essential for the rational design. It has been reported that the thermal stability of enzymes was increased by various strategies such as the introduction of additional disulfide bonds (Jeong, Kim, Yun, Choi, & Cho, 2007), (Le, Joo, Yoo, & Kim, 2012), replacement of proline for some critical locations (Nakamura, Tanaka, Yada, & Nakai, 1997), (Tian et al., 2010), (Wang et al., 2014) or rigidifying the flexible residues (H. Kim, Le, & Kim, 2010), (Badiyan et al., 2012), the introduction of salt bridges (Chan, Yu, & Wong, 2011). In this review, some successful examples of rational protein design focusing on engineering disulfide bonds and the introduction of proline to enhance the thermostability of enzymes applying in the industrial sector will be presented.

**Table 1**

Industrial applications of enzymes

Enzymes	Applications	References
Lipase B from <i>Candida antartica</i> (CalB) (Novozyme 435) and other lipases	Production of Biodiesel	(Akoh et al., 2007; Ko'se et al., 2002; Shimadaa et al., 1999).
Lipase B from <i>Candida antartica</i> (CalB) (Novozyme 435)	Production of Glycerol carbonate (a key multifunctional compound for chemical intermediate and cosmetic solvents)	(S. Kim et al., 2007).
Lipase B from <i>Candida antarctica</i> (CalB)	Production of poly(D-lactide), a biodegradable bioplastic	(M. Hans, Keul, & Moeller, 2009)
Lipase B from <i>Candida antartica</i> (CalB) (Novozyme 435)	Production of Gamma-Butyrolactone Methacrylate (GBLMA) for production of the photoresist resin	(S. C. Kim, Nam, Kim, & Song, 2010)
Xylanases	Degradation of hemicellulosic biomass for biofuel synthesis and pulp industry	(Saha, 2003), (Wang et al., 2014)
Alpha-amylase family	Enzymatical hydrolysis of starch to product maltodextrin, modified starch or glucose and fructose syrup	(Maarel et al., 2002)
D-xylose isomerase from <i>Streptomyces diastaticus</i> No.7 (M1033)	Converting xylose into xylulose and catalyze glucose into sweeter fructose. This is widely used in the food	(Zhu et al., 1999)

Enzymes	Applications	References
	industry for high fructose corn syrup (HFCS)	
Organophosphate hydrolase from <i>Pseudomonas diminuta</i>	Detoxification of harmful organophosphate pesticides	(Farnoosh, Khajeh, Latifi, & Aghamollaei, 2016)
Acetylcholinesterase from <i>Drosophila melanogaster</i>	Biosensor for detection of organophosphate and carbamate insecticides	(Siadat, Lougarre, Lamouroux, Ladurantie, & Fournier, 2006)
GH5 cellulases	Production of cellulosic ethanol from lignocellulose	(Badiyan et al., 2012)

Source: The researcher's data analysis

## 2. Rational protein engineering through the introduction of a proline

Introduction of proline has been successfully applied to increase the thermal stability of several enzymes such as neutral protease from *Bacillus stearothermophilus* (Nakamura et al., 1997), D-xylose isomerase from *Streptomyces diastaticus* No.7 (M1033) (Zhu et al., 1999), liquefying  $\alpha$ -amylase from *Bacillus* sp. Strain KSM-1378 (Igarashi et al., 1999), methyl parathion hydrolase from *Ochrobactrum* sp. M231 (Ochr-MPH) (Tian et al., 2010), and xylanase from *Streptomyces* sp. strain S9 (Wang et al., 2014). Proline, which possesses a special cyclic structure of the side chain, restricts backbone configuration and hence decrease backbone configurational entropy compared to other amino acids (Watanabe & Suzuki, 1998). The general rule for engineering proline was proposed by Suzuki based on their discovery that the increase in proline number in second positions of b-turns of *Bacillus* oligo-1,6-glucosidases with their thermostability (Suzuki, 1989, 1999). The presence of proline at b-turns increases protein's thermostability mainly because of a decrease in the backbone entropy of unfolding (Igarashi et al., 1999). In rational design, the selection of suitable residues for site-directed mutagenesis plays the central role. To engineer the enzyme belonging to a family with different thermal stability, multiple sequence alignments to identify the critical residues which conserved in higher thermal stability, but not in less thermal stability is a good strategy for engineering through the introduction of proline. The combination of the introduction of proline and multiple sequence alignment significantly increased 2.5-fold in half-life at 35°C for D-xylose isomerase from *Streptomyces diastaticus* No.7 (M1033) (Zhu et al., 1999) and 2.6-fold in half-life at 50°C for a liquefying  $\alpha$ -amylase from *Bacillus* sp. Strain KSM-1378 (Igarashi et al., 1999) (Table 2). Protein engineering for target enzyme with limited available information, however, multiple sequence alignment does not work. Residual flexibility analysis based on Molecular Dynamics (MD) Simulations could be an excellent choice. Methyl parathion hydrolase from *Ochrobactrum* sp. M231 (Ochr-MPH) mutant exhibited a 3.3°C increase in melting temperature ( $T_m$ ) and a 5°C increase in  $T_{50}^{10}$  (the half-survival temperature at 50°C for 10 minutes) compared to the wild type (Tian et al., 2010). In addition, the combination of flexibility analysis using MD simulations and multiple sequence alignment to increase >9-fold in half-life at 70°C and 7.0°C in  $T_m$  for xylanase from *Streptomyces* sp. strain S9 (Wang et al., 2014). The successful researches demonstrated the usefulness of the introduction of proline on the

thermostability of a given enzyme. However, the introduction of proline does not always get beneficial results. It was reported that the introduction of proline at the N-terminal of the helix region led to an increase in enzyme's thermostability, while the introduction of proline in the middle of the helix region led to a decrease in the enzyme's thermostability (Tian et al., 2010). The selection of suitable positions for proline substitution is critical to the success of engineering.

### 3. Rational protein engineering of disulfide bonds in proteins

The engineering of disulfide bonds in protein by introducing an additional disulfide bond(s) into proteins is another promising strategy for improving the thermostability of industrial enzymes. Disulfide bonds, which are additional covalent linkages in a polypeptide sequence and occur naturally in secreted proteins, contribute significantly to protein's stability (Wells & Powers, 1986), (Petsko & Ringe, 2004), (Siadat et al., 2006). The disulfide bonds contribute to protein's stability caused mainly by decreasing entropy of the unfolded form of protein (Anfinsen & Scheraga, 1975) or decreasing the unfolding rate of irreversibly denatured protein (Clarke, 1993), (Plaza, Ibarra-Molero, & Sanchez-Ruiz, 2000). Introducing disulfide bonds into protein demonstrated its usefulness to increase the thermal stability of several enzymes such as ribonuclease H from *Escherichia coli* (Kanaya et al., 1991), xylanase from *Bacillus stearothermophilus* No. 236 (Jeong et al., 2007), Acetylcholinesterase from *Drosophila melanogaster* (Siadat et al., 2006), and lipase B from *Candida antarctica* (Le et al., 2012).

The engineering of protein's thermostability by introducing additional disulfide bonds include two important steps. The first step is to predict residue pairs having the possibility to form disulfide bonds in a protein. The second step is to select promising residue pairs among the predicted residue pairs for site-directed mutagenesis into cysteines having high potential to increase protein's thermostability. In the first step, the residue pairs having the possibility to form disulfide bonds were usually identified by several automated prediction programs such as modeling of disulfide bonds in proteins (MODIP) (Dani, Ramakrishnan, & Varadarajan, 2003), (Sowdhamini et al., 1989) or Disulfide by Design (DbD) (Dombkowski, 2003). Different programs have different criteria to predict the residue pairs having the possibility to form disulfide bonds. MODIP evaluates geometrical parameters (dihedral angles and S-S distance) of model disulfide bonds from each residue pairs and classified it into grade A or B or C depending on their geometrical satisfaction (Dani et al., 2003), (Sowdhamini et al., 1989). On the while, DbD evaluates the formation energy of model disulfide bonds with fixed geometrical parameters ( $C_b-S_g$ ,  $S_g-S_g$  bond lengths and  $C_b-S_g-S_g$  bond angles are 1.81 Å, 2.04 Å and 104.15°, respectively). A residue pair that generated a lower energy value for a putative disulfide bond has a higher possibility to form a disulfide bond (Dombkowski, 2003). The thermal stability of several enzymes was significantly increased by applying MODIP and/or DbD such as indole glycerol phosphate synthase by MODIP (Ivens et al., 2002) and xylanase from *Bacillus stearothermophilus* No. 236 by DbD (Jeong et al., 2007) and lipase B from *Candida antarctica* by both MODIP and DbD in our previous study (Le et al., 2012). The successful researches that have applied MODIP and DbD to increase the thermal stability of enzymes demonstrated the usefulness of those computational tools to predict the possible disulfide bonds in proteins. However, both MODIP and DbD, which are focus only on identifying residue pairs having

possibility to form disulfide bonds, do not have any tool to evaluate potential to increase the thermostability of genetically engineered protein bearing the newly disulfide bond(s). Additionally, the number of the residue pairs which could form new disulfide bonds suggested by MODIP and/or DbD is relatively large for site-directed mutagenesis (Le et al., 2012). Therefore, the efficient selection of promising residue pairs for the site-directed mutagenesis is crucial for time-saving and cost-effective engineering of disulfide bonds in improving the thermostability of target enzymes. Different selection strategies to select the promising residue pairs for site-directed mutagenesis were introduced and got success in improving the thermostability of several enzymes. The MODIP-predicted residue pairs for disulfide bond formation were further filtered for experimental mutagenesis by simply selecting the residue pairs which are localized nearby the active site of the enzyme and/or may lead to short-range disulfide bond formation of indole glycerol phosphate synthase (Ivens et al., 2002). Multiple sequence alignment having the ability to identify the conserved residues and critical variants from the protein family has shown its usefulness for selection of the promising residue pairs in protein engineering of disulfide bonds of enzymes belonging to a family with available information on their diverse thermostability such as xylanase from *Bacillus stearothermophilus* No. 236 (Jeong et al., 2007) and acetylcholinesterase from *Drosophila melanogaster* (Siadat et al., 2006). Twenty-five residue pairs having the possibility to form the disulfide bonds in xylanase from *Bacillus stearothermophilus* No. 236 by DbD program, for example, were further filtered by multiple sequence alignment with other 11 family xylanases and select only one residue pair which are evolutionarily conserved cysteines for site-directed mutagenesis. The mutant with one additional disulfide bonds from the residue pair increased 5°C in half-survival temperature after 20 minutes and increased 3.5-fold in half-life inactivation at 65°C compared (Jeong et al., 2007). For the development of enzymes that do not belong to a family with diverse thermostability or have little information about their family or enzymes showing too many possible residue pairs for disulfide bond formation from MODIP and DbD, a general tool to identify the promising residue pairs to increase enzyme's thermostability was developed. Residual flexibility analysis which was firstly proposed by our group is one example of a general selection tool for selecting the promising residue pairs from the predicted programs. The flexibility analysis of the predicted residue pairs by using both B-factor values from enzyme's crystal-structure and FRODAN dynamics for given residue pairs before and after *in-silico* mutagenesis was used for selection of the promising residue pairs for experimental site-directed mutagenesis in engineering disulfide bonds of lipase B from *Candida antarctica* in our previous study (Le et al., 2012). The B-factor values, the atomic displacement parameters determined by high-resolution X-ray crystallographic studies represent the smearing of atomic electron densities around the equilibrium positions due to thermal motion and positional disorder (Parthasarathy & Murthy, 2000). Beside B-factor values which indicates the flexibility of static states and flexibility motions which predict the flexibility changes of local structures is also essential for evaluation and selection of the residue pairs for site-directed mutagenesis (Joo, Pack, Kim, & Yoo, 2011), (Le et al., 2012). In addition, energy stability evaluation based on MD Simulations was also used as a general tool for the selection of the promising residue pairs in engineering thermostability of organophosphate hydrolase from *Pseudomonas diminuta* (Farnoosh et al., 2016). The rational introduction of disulfide bonds into proteins has demonstrated its usefulness to enhance the thermal stability of many industrial enzymes.

However, the introduction of disulfide bonds does not always get the expected results. Destabilization of protein's stability through introduction of additional disulfide bonds has been reported in one or some mutants of several enzymes such as T4 lysozyme (Wetzel, Perry, Baase, & Becktel, 1988), acetylcholinesterase from *Drosophila melanogaster* (Siadat et al., 2006), and lipase B from *Candida antarctica* (Le et al., 2012).

**Table 2**

The computational protein engineering to enhance thermostability of enzymes

Engineering strategies	Target Enzymes	Computational methods	Improved property of mutant	References
Introduction of proline	Neutral protease from <i>Bacillus stearothermophilus</i>	Engineering the alpha-helix (residues 140-153) which combines N- and C-terminal domains in the active sites of the enzymes.	Increased 7.5 °C in half-survival temperature after 30 minutes. increased 4.4 ° in T <sub>m</sub>	(Nakamura et al., 1997)
Introduction of proline	D-xylose isomerase from <i>Streptomyces diastaticus</i> No.7 (M1033)	Multiple Sequence alignment of the enzyme with other glucose isomerases of thermophilic bacteria.	Increased 2.5-fold in half-life inactivation at 35 °C	(Zhu et al., 1999)
Introduction of proline	Liquefing $\alpha$ -amylase from <i>Bacillus</i> sp. Strain	Multiple sequence alignment of LAMY with	Increased 4 °C in half-survival temperature	(Igarashi et al., 1999)

Engineering strategies	Target Enzymes	Computational methods	Improved property of mutant	References
	KSM-1378 (LAMY)	alpha-amylases from other <i>Bacillus</i> sp.	at 50 °C after 10 minutes and 2.6-fold increased in half-life at 50°C.	
Introduction of proline	Methyl parathion hydrolase from <i>Ochrobactrum</i> sp. M231 (Ochr-MPH)	MD Simulations to find the greatest conformation fluctuation.	Increased 5 °C in $T_{50}^{10}$ (the half-survival temperature at 50 °C after 10 minutes) and 3.3 °C in $T_m$	(Tian, et al., 2010)
Proline or glutamic acid replacement	Xylanase from <i>Streptomyces</i> sp. strain S9	Multiple-sequence analysis and molecular dynamics simulations predicted 4 residues affecting the	Increased >9-fold increase in half-life at 70°C and 7.0 °C higher in $T_m$	(Wang et al., 2014)



Engineering strategies	Target Enzymes	Computational methods	Improved property of mutant	References
		thermostability of the xylanase.		
Introduction of disulfide bonds	Acetylcholinesterase from <i>Drosophila melanogaster</i>	<b>Prediction:</b> Choosing a residue pair that has their C <sub>β</sub> the distance of 3.6 - 4.0 Å. <b>Selection:</b> Multiple sequence alignment.	Increased 170-fold in half-life at 50 °C.	(Siadat et al., 2006)
Introduction of disulfide bond	Xylanase from <i>Bacillus stearothermophilus</i> No. 236	<b>Prediction:</b> Disulfide by Design. <b>Selection:</b> Multiple sequence alignment	Increased 5 °C in half-survival temperature after 20 minutes and increased 3.5-fold in half-life inactivation at 65 °C.	(Jeong et al., 2007)
Introduction of disulfide bond	Lipase from <i>Rhizomucor miehei</i>	<b>Prediction:</b> Disulfide by Design.	Increased 5-fold in half-life	(Z. Han, Han, Zheng, & Lin, 2009)

Engineering strategies	Target Enzymes	Computational methods	Improved property of mutant	References
		<b>Selection:</b> Selecting the residue pairs in the lid region which are reported to be related to activation of the enzyme.	inactivation at 60 °C	
Introduction of disulfide bonds	Cellulase C (a member of GH5 endoglucanases)	<b>Prediction:</b> Disulfide by Design <b>Selection:</b> MD Simulations analysis at various temperatures	Increased 4 °C in half-survival temperature after 10 minutes and increased >5-fold in half-life at 65 °C.	(Badieyan et al., 2012)
Introduction of disulfide bonds	<i>Candida antarctica</i> lipase B (CalB)	<b>Prediction:</b> MODIP and Disulfide by Design. <b>Selection:</b> Residual flexibility analysis which	Increased 8.5 °C in half-survival temperature at 50 °C for 60 minutes	(Le et al., 2012)

Engineering strategies	Target Enzymes	Computational methods	Improved property of mutant	References
		combines B-factor values of residues and FRODAN dynamics	and a 4.5-fold increase in half-life at 50 °C.	
Introduction of disulfide bonds	Organophosphate hydrolase from <i>Pseudomonas diminuta</i>	<b>Prediction:</b> Disulfide by Design. <b>Selection:</b> Stability energy evaluation using ERIS database and molecular dynamics Simulation (i.e., Gromacs).	Increased 1.5-fold in half-life at 65 °C, but it only remained 30% catalytic efficient (kcat/Km).	(Farnoosh et al., 2016)

Note: Tm: Melting temperature is a temperature at which protein remains 50% of its native structure

Half-life (t50): The time at which protein remain 50% its activity at given temperature

Source: The researcher's data analysis

#### 4. Conclusion and perspectives

Rational protein engineering has demonstrated its usefulness to successfully increase the thermostability of various industrial enzymes. Some mutants from rational protein engineering also exhibited a decrease in thermostability. Identification of the promising residues for experimental site-directed mutagenesis is critical for the success of the engineering and dependent both on the enzyme and applied computational strategies. Several protein-stabilizing factors such as disulfide bonds and proline residues at critical positions were applied to increase the thermal stability of the enzymes by the support of several computational strategies such as multiple sequence alignment and residual flexibility. Multiple sequence alignment is very helpful and limited in the engineering of a given enzyme belonging to a family with available information on their diverse thermostability. More general and direct computational tools such as FRODAN dynamics and MD Simulations to evaluate residual

fluctuation and energy stability have been increasingly used to work individually or collaboratively with simpler computational tools such as multiple sequence alignment for engineering the thermostability of enzymes. In addition, searching for better and simpler computational strategies would be beneficial to get better thermal stability of the enzymes with lesser effort.

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