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Review

Enhancing thermostability of the biocatalysts beyond their natural function *via* protein engineering

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Majority of the naturally occurring enzymes lacks essential features required during the harsh conditions of the industrial processes, because of their less stability. Protein engineering tool offers excellent opportunity to improve the biochemical properties of these biocatalysts. These techniques further help in understanding the structure and function of the proteins. Most common methods employed in protein engineering are directed evolution and rational mutagenesis. Several research groups have utilized these methods for engineering the stability/activity of diverse class of enzymes. *In silico* tools further plays an important role in designing better experimental strategy to engineer these proteins. The availability of vast majority of data on protein thermostability will enable one to envisage the possible factors that may contribute significantly in maintaining the protein structure and function during various physical conditions. This review discusses the common method employed in protein engineering along with various molecular/computational approaches that are being utilized for altering protein activity, along with important factors associated with these processes.

Key words: Computational database, rational, directed evolution, thermostability, hydrophobicity, three dimensional structure, configuration, biocatalysts.

INTRODUCTION

Almost all industrial processes are carried out at high temperature; therefore there is pressing need to discover new thermostable enzymes/or modify existing enzymes, using protein engineering approach. Thermostable enzymes are widely employed during manufacturing of detergents, food processing, production of high fructose corn syrup etc. (Crabb and Mitchinson, 1997). Previously, Turner et al. (2007) has also provided a valuable insight into the use of thermostable enzymes in biorefining. Industrial processes performed at high temperature possesses following major advantages that is, increased rate of reaction, less microbial contamination, increased solubility of the substrates etc. A recent progress made in protein engineering approach has resulted in modification of several biocatalysts for enhanced thermostability. Two most common methods that are employed *in vitro* evolutions of thermostable enzymes are: directed evolution, which do not require any structural or mechanistic information and rational designing, that require prior knowledge of the three dimensional structure, as depicted in the flow chart (Figure 1).

A number of factors are associated with protein thermostability and mainly include increased hydrogen bonding, increased percentage of hydrophobic residues and less percentage of thermo labile residues etc. (Sadeghi et al., 2006; Russell et al., 1997; Bogin et al., 1998; Kumar et al., 2000). Among these factors, the hydrophobicity in protein is known to direct configurationally complexities of folding and unfolding (Kauzmann,

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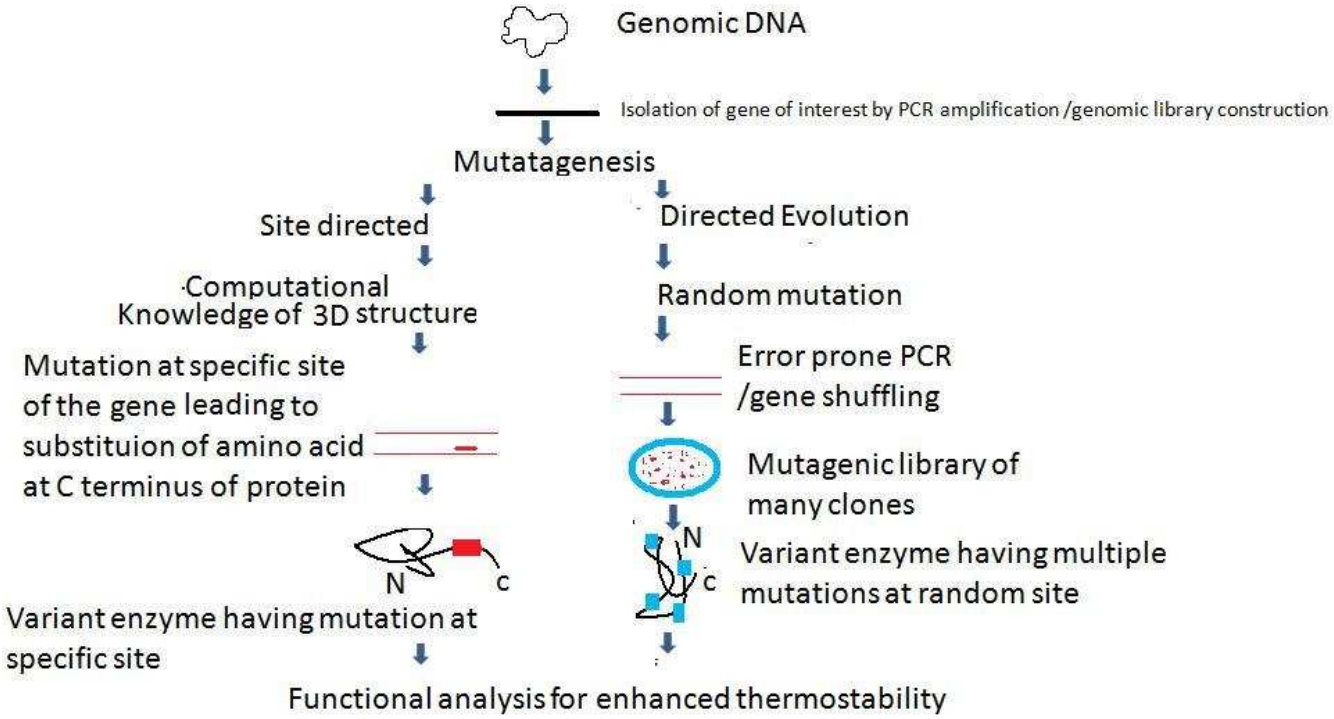

Figure 1. Flow chart representing methodology involved in mutagenesis.

1959; Franks, 2002). In addition to this, stability of a folded protein is modulated by weak interactions that is, vander Waals interactions, H-bonding, salt bridges, aromatic-aromatic interactions and disulfide bonds etc. (Feller, 2010). Interestingly, despite difference in protein sequences and structures of extremophiles and its counterpart mesophile, there is only marginal difference in their free energy of stabilization. Therefore, no general strategy of stabilization has yet been established for specifying a structure that is carrying a particular function (Jaenicke and Bohm, 1998; Leisola and Turunen, 2007). In this review, we are primarily discussing methods (directed evolution/rational/computational) in modifying industrially relevant enzymes for enhanced thermostability. This review further shed light into various factors responsible for enhanced thermostability.

DIRECTED EVOLUTION

Directed evolution is routinely used by several research groups in creating enzymes with improved properties. Directed evolutions methods that is, error prone polymerase chain reaction (PCR) and gene shuffling generate arbitrary mutations, and resulted in positive/negative or neutral mutations. Despite lack of prior knowledge of the structure, the mutations obtained using these methods has produced a number of thermostable enzyme variants. Here, we have provided few examples such as, p-nitrobenzylesterase (Spiller et al., 1999), subtilisin E (Zhao and Arnold, 1999; Miyazaki et al., 2000), lipase (Ahmad et al., 2008), N-carbamyl-D-amino acid amidohydrolase (Yu et al., 2009), histone acetyltransferase (Leemhuis et al., 2008), asparaginase (Kotzia and Labrou, 2009) amylase (Kim et al., 2003). Interestingly, thermostabilizing mutation created by directed evolution sometime resulted in rare mutation, and cannot be rationalize structurally. Never the less, these mutations contribute a lot in understanding structure and function of the enzymes.

Furthermore, data gathered from several studies point out that mutation at protein surface are important in enhancing thermostability. Below, we are discussing few examples where mutation at surface has resulted in enhanced protein thermostability. An interesting example is generation of a variant subtilisin E from mesophilic bacteria *Bacillus subtilis* that showed remarkable increase in its optimum temperature (approximately 17℃) compared to WT. Moreover, the variant protein showed identical optimum enzyme activity, as observed with thermophilic homologue thermitase, produced by *Thermoactinomyces vulgari* (Zhao and Arnold, 1999). In another case study, seven arbitrary mutations were created in a maltogenic amylase by deoxyribonucleic acid (DNA) shuffling which enhanced optimum temperature of the enzyme by 15℃, compared to wild type enzyme. O ut of these, the mutations A398V and Q411L stabilized the enzyme by enhancing inter domain hydrophobic

interactions, while other two mutations R26Q and P453L resulted in increased H bonding (Kim et al., 2003).

RATIONAL APPROACHES

Here, prior knowledge of the three dimensional structures is prerequisite for selecting useful mutations. There are number of rational for designing useful mutations, we are discussing few examples, first case where improved residual packing of protein structure (lipase) of a *Bacillus subtilis* resulted in enhanced protein thermostability. Here, glycine and alanine residues were targeted to improve packing of protein structures and consequently site directed mutagenesis was done to generate variants A38V, G80A and G172A. Interestingly, the packing at the interior of the protein enhanced the stability (Abraham et al., 2005). In second case, twelve mutations were created in lactate oxidase, based on following parameters; mutation that decrease enthalpy and increase free energy of difference between folded and unfolded state, changing amino acids at the interface, which can resist irreversible denaturation, replacement of hydrophobic pocket residues, for example, alanine to valine/leucine.

So far, out of these twelve deliberately designed rational mutations, only one had a positive effect on thermostability (Kaneko et al., 2005). Nevertheless, several other mutations were created by considering number of factors that had improved thermostability of mutant protein to greater extent than the wild type, for example, entropic stabilization by mutating Gly→Ala, X-Pro (Matthews et al., 1987), enhanced secondary structure propensity (Zhao and Arnold, 1999), increased H-bonding (Kim et al., 2003), hydrophobic interactions (Song and Rhee, 2000), electrostatic interactions (Blasco et al., 2000), helix capping by introducing residues that interact with α- helix dipole (Nicholson et al., 1988, 1991), disulfide bridges (Wang et al., 2006), salt bridges (Serrano et al., 1990), aromatic interactions (Burley and Petsko, 1985).

DIRECTED/RATIONAL

It is worth to discuss here that designing thermostable mutations rationally is difficult, because many of these are neutral and destabilizing. Additionally, despite several efforts, no universal strategy could be generalized for creating stable mutations. Furthermore, the molecular understanding of proteins is narrow and predicting mutation on rational basis is not so robust (Kaneko et al., 2005). On the other side, though the success of getting a desired mutation is less in directed evolution strategy, this technique has resulted in evolution of favorable mutations that further enriched understanding of structure and function of a protein to some extent and can also direct in scheming rational mutation. The major limitation for directed evolution approach is lack of efficient screening for selection of desired variants.

COMPUTATIONAL METHODS IN PREDICTING STABILITY (SEQUENCES/STRUCTURE)

Computational methods have contributed significantly in designing effective and favorable mutations (Kraemer-Pecore et al., 2001; Gordon et al., 1999). With the rapid expansion of structural bioinformatics (RCSB) and protein data bank (PDB), there will be enormous benefit for structural engineer in creating vast libraries of desired variants (Johannes and Zhao, 2006). In addition, a number of software have been developed which can predict structure of the thermostabilizing mutation (Dantas et al., 2003). Furthermore, software like homology derived secondary structure of proteins (HSSP) is useful computational tool that distinguish conserved and variable regions of sequential database. On specifying PDB code, it scans multiple sequences and identifies evolutionary conserved amino acid positions which are projected at three dimensional levels (Patrick and Firth, 2005). The methods were employed previously in predicting three mutations with a modeled enzyme that improved melting temperature of protein by 10℃ and half life of the protein to 30 fold at 50° C (Korkegian et al., 2005).These structural predictions can efficiently be used in designing high temperature adapting mutations. Computational analysis of protein structures is very promising in designing mutations. Basu and Sen (2009) also suggested the use of computational tools for designing thermostabilizing mutations. The structure based protein designing had successfully been used for producing a thermostable papain (a plant cysteine protease) by performing mutations in the inter domain region, to enhance H-bond, salt bridge interactions and to reduce the flexibility. These all factors contributed significantly in enhancing the thermostability of papain mutant whose half life was extended by 94 minutes at 60℃ (Choudhury et al., 2010). In summary, for designing thermo stabilizing mutations, physical interactions among residues at atomic level should be optimized, while steric constraints should be avoided during substitution. Recently, a fully automatic protocol (Rosetta $_{VIP}$) was developed which can select point mutation that improve the quality of core packing and may provide enhanced thermodynamic stability to the protein (Borgo and Havranek, 2012).

ROLE OF HYPERTHERMOPHILLIC GENES/ HOMOLOGUES IN PREDICTING STABLE MUTATIONS

Alternatively, thermostable proteins can be generated by

introducing conserve amino acid residues from their ancestral hyperthermophilic homologues. Let us discuss few such examples where this approach was utilized successfully for obtaining thermostable proteins for example, a thermostable isocitrate dehydrogenase from *Cladococcus noboribetus* (Iwabata et al., 2005) and isopropyl malate dehydrogenase from *Thermus thermophilus* (Watanabe et al., 2006). In another case, several ancestral residues were incorporated in β-amylase of a *Bacillus circulans* which enhanced the thermostability of mutant protein compared to wild type amylase (Yamashiro et al., 2010). In one interestingly example, a mesophilic triosephosphate isomerase was converted into a super stable enzyme, without losing its catalytic power on replacing its highly conserved residues (Williams et al., 1999). During *in vitro* evolution of *Bacillus* lipase (lip A) a remarkable 15℃ shift in melting temperature and approximately 20℃ shift in thermal denaturation was observed.

Interestingly, multiple sequence alignment of this lipase suggests that all these mutations were present in at least one of its natural homologue (Ahmad et al., 2008). In another case study, a lipase with six mutations was generated from *Bacillus* by replacing glycine to valine/alanine. Out of these six, three mutants (A38V, G80A, and G172A) showed enhanced stability and were found to be conserved (Abraham et al., 2005). Random evolution methods in combination with semi rational consensus approach generated a thermostable xylanase XT6 from *Geobacillus stearothermophilus* (Zhang et al., 2010). Recently, we also reported one thermostable mutant where the altered amino acid of metagenomic lipase replaces conserved amino acids (Sharma et al., 2012). Hence, it becomes evident from aforementioned examples that laboratory evolved thermostable enzymes in majority of the cases, substituted those residues which were already existed in their natural homologues and may be helpful in predicting thermostable mutations.

MAJOR FACTORS CONTRIBUTING TOWARDS STABILIZING MUTATIONS

For successful engineering of thermostable proteins, we need first to understand the factors associated with the thermostability. Interestingly, the structural characterization of several thermo stabilizing mutations revealed that they are generally affected by secondary structure, solvent accessibility of the important residues and hydrophobicity profile of a protein. Below, we attempted to shed light on controlling role of these factors in protein stability.

1. Thermostability of a protein is highly influenced by secondary structure that is, helix, sheet, coil or random turns. Previous studies have shown that most remarkable thermostabilizing mutations generally lie in the random coils, turns and loops. On the other side, the helix and sheets are less cooperative for such substitutions, may be because of less plasticity in these regions. Some of the studies carried out previously reflect that most of these mutations are present in the loop and random coils, e.g. mutation G195E in galactose oxidase (Sun et al., 2001) lipase (Acharya et al., 2004; Ahmad et al., 2008) subtilisin E (Zhao, 1999) horse radish peroxidase (Ryan and O'Fágáin, 2008), α glucosidase, (Zhou et al., 2010), L-asparaginase (Kotzia and Labrou, 2009), *N*-carbamyl-D-amino acid amidohydrolase (Yu et al., 2009).

2. Next, the presence of hydrophobic residues at the interior of proteins helps in maintaining the structural integrity. They determine the folding process of protein (Dill, 1990), interestingly, sequence alignment from various proteins revealed that the hydrophobic core regions in proteins is conserved throughout evolution, therefore suggesting possible role of these residues in maintaining stability and function (Di et al., 2003). The conserve nature of the proteins at the interior may be attributed to low substitution rate due to selective pressure (Lim and Sauer, 1989, 1991; Smith and Raines, 2006). An example is ubiquitin, where random mutation has revealed that specific core packing arrangements are critical for maintaining stability (Finucane and Woolfson, 1999). Furthermore, most of the residues essential for function of a bovine pancreatic ribonuclease (RNase A) were found buried in hydrophobic core (Smith and Raines, 2006). In another study, out of 15 serine residues of an RNase, only one that is, Ser75 found to be buried, conserved and critical for stability (Johnson et al., 2007). Many studies have proven that mutation within core of proteins are destabilizing, because of formation of cavity that resulted in loss of vander waals interactions (Xu et al., 1998; Ratnaparkhi and Varadarajan, 2000; Kono et al., 2000; Chakravarty et al., 2002; Vlassi et al., 1999; Beadle and Shoichet, 2002).

3. It is further suggested that surface residues have high solvent accessibility relative to average residue of protein and contributes significantly in stabilization (Leemhuis et al., 2008). Replacing a charged residue at the protein surface must fit criteria of optimized local interactions, while minimizing repulsive interactions; otherwise such substitution may result in destabilization of the protein structure, as observed in ribonuclease H1 of the *E. coli* (You et al., 2007). Recently, a cellobiohydrolase from *Talaromyces emersonii* (*Te* Cel7A) showed enhanced protein thermostability, when five disulphide bonds were engineered at surface of the protein (Voutilainen et al. 2010). Furthermore, two human acylphosphatase enzymes (AcPh and Cdc42 GTPase) were made thermostable by bringing in residues that increase the surface charge interactions (Gribenko et al., 2009). Typically, polar residues are preferred at protein surface; however current studies pointed out that even hydrophobic interaction can contribute to stability for example, on replacing a charge residue with hydrophobic

one (arginine) at the surface of helical protein, enhances protein stability (Spector et al., 2000), another such example is enhancement of thermostability in family 11 of xylanases from *Bacillus subtilis* (Miyazaki et al., 2006). Briefly, such an observation may be attributed to flexibility of protein at the surface.

CASE STUDIES FROM OUR LABORATORY

Our laboratory is working in field of directed evolution/site directed mutagenesis for the past 10 years. Recently, we reported a highly thermostable lipase mutant (Sharma et al., 2012). The mutation was observed on the surface of protein having substitution N355K. Interestingly, the mutant lipase showed 144 fold enhancement in the thermostability compared to WT lipase. Moreover the catalytic efficiency was also enhanced 20 folds. During biophysical characterization, we found that mutant retained its secondary structure at high temperature compared to wild type enzyme, simultaneously, the molecular dynamics of the generated three dimensional structure revealed increase in the hydrogen bonding. In another case, Khurana et al. (2010) reported a mutant of a *Bacillus* lipase that showed enhanced half of the mutant 3 folds at 50℃. The kinetic parameters of the muta nt enzyme were significantly altered. The mutation was observed on the part of helix which is exposed to the solvent and away from the catalytic triad. This study demonstrated that replacement of a solvent exposed hydrophobic residue (Ile) in WT to a hydrophilic residue (Thr) in mutant might impart thermostability to the protein.

CONCLUSION

Biochemical nature of proteins is largely determined by number of weak non-covalent interactions which make stability of protein highly unpredictable. Thermostable variants generated by directed evolution and rational approaches can cast light into molecular behavior of proteins. Consequently, with the availability of massive sequence, structure database and *in silico* tools along with robust molecular techniques, it is becoming easy for protein biochemists to alter the function of the biocatalysts beyond their natural functions. Furthermore, the protein engineering hold promises to unravel the mechanism underlying biochemical and molecular function of proteins.

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