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Review Article

Recent Advances in Structure-Based Protein Engineering

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Abstract: The last decade has seen an exponential increase of protein structures solved by X-ray crystallography, NMR and cryo-electron microscopy. The existing information on the protein crystal structure and various computational design toolboxes are furnishing protein engineering more accurately than ever. Structure-based protein engineering involves the application of structural knowledge and software tools to alter protein structures and functions. Much work has been focused on enzyme structure analysis by computational tools to detect key residues responsible for specific properties. We observe that structure-based engineering techniques are potential and compatible approaches that greatly simplify the process of improving certain properties of enzymes. The molecular modeling and *de novo* design tools are instrumental in increasing antibody stability and binding affinity for therapeutics. They are also proven helpful in identifying epitope of antigens for vaccine development. As the recent advances in structure-guided protein engineering are summarized in the review, it is clear that the structure-based protein engineering could result in tremendous saving in time and investment compared to traditional methods.
Keywords: rational design, computational design, enzyme engineering, epitope prediction, antibody engineering.

INTRODUCTION

Protein engineering is the process by which proteins are modified for the desirable properties. Native proteins are adapted to their specific functions in a cell, but often they are poorly suited to meet the needs of various industrial applications such as temperature[1], pH[2] and salinity. Recently, protein engineering has become a very attractive research area due to its importance in understanding protein structure-function relationships, protein-protein interactions and increasing the industrial applicability of enzymes[3].

Protein engineering prospects, including the roles of chemical synthesis of DNA, x-ray crystallography, and computational modeling of protein structures have been discussed by Ulmer. The researcher demonstrated first that, by combining information on artificial gene synthesis and crystal structures, different properties of proteins can be modified[4]. During the last 20 years, there has been a continuous flow of reports describing significant developments in the subject area[5]. Commonly used protein engineering methods include rational design and directed evolution. The choice of method therefore is still a case-to-case decision, depending on the existing structural, mechanistic knowledge and the particular interest of researchers as each of the strategy has some advantages and disadvantages (Table 1). The rational design is often a structured-based strategy. On the other hand, directed evolution does not require information about protein structure-function relationship[6]. Sometimes researchers applied both rational and directed evolution together[7]. This engineering method is called semi-rational approach for which structure is partially required (Fig. 1C). The present study will not discuss the semi-rational approach as it is not completely structured-based method.

Recently, the availability of protein structure has been broadening the opportunity to modify proteins for desirable traits or to make new ones by structure-based engineering approaches. About 91960 proteins and 4654 protein-nucleic acid complex structures are available in Protein Data Bank (PDB) until April 8, 2014. The wealth of information about protein structures has drawn great attention from researchers around the world, which has opened a new skyline in structural protein engineering. As each protein family has at least one structure available now, the homology modeling is much more accurate than before.

Nowadays, homology modeling has become a powerful method to recognize desired residues in the homologous proteins among a certain protein family. Current review first covers the common structure-based protein engineering strategies, which summarizes recent advances and future prospects. Then various examples are presented for the protein engineering of enzymes to increase stability, substrate- and cofactor specificities. At last, we will describe some recent achievement of structural protein engineering approach applied for pharmaceutical purposes.

PROTEIN ENGINEERING STRATEGIES

Simple rational design strategy based on the known protein structures

Simple rational design involves the straightforward analysis of protein structures to find the mutation sites for desirable traits. Due to its simplicity, it is the most frequently used method for protein engineering. A specific stepwise method for simple rational design is illustrated in Figure 1(A). Nixon and Firestine mentioned three sequential steps for the process of simple rational design[8]. These steps are (a) choice of appropriate scaffold, (b) identification of specific residue to change and (c) selection of desirable mutants by characterization. In the first stage, suitable scaffold can be chosen based on the knowledge of scaffold structure or a homologous scaffold structure. This structural knowledge is used to identify specific amino acid residue for mutations, because the region seems to be a crucial point that can largely affect the protein properties for desired reactions[9].Selection of targeted amino acid for mutation can be done by three dimensional (3D) structure analyses. Using these approaches, Yang and co-workers improved oxidative stability of alkaline amylase from *Alkalimonas amylolytica* by structure-based engineering of methionine residues located in the catalytic cores of the active site[10].Five methionine (M) residues (M145, M214, M229, M247 and M317) were targeted to replace with leucine (L), as methionine residues are oxidation prone that could result in increasing side chain sizes and the steric obstruction of the active site. After such mutations, researchers screened for desirable mutants and observed that the M145L, M214L, M229L, M247L, and M317L mutants retain 28%, 46%, 28%, 72%, and 43% of the original activity, respectively. In addition, compared to wild type enzyme, the pH stability of M247L is extended from 6 to 12, while the pH stability for the rest of mutants remains unchanged.

More advanced rational design techniques involving extensive usage of computational techniques

The structure-based rational requires detailed information about the target protein 3D structure. When the structure of target or homologous protein is on hand, a more distinct structure-function relationship can be analyzed [9].Lim and co-workers engineered a structural monomer of 13 kDa, mSA, by combining rhizavidin and streptavidin (SA)sequences, which is stable but binds biotin with low nanomolar affinity[11]. Concurrently, another research group designed the monomer by homology modeling to improve its binding kinetics. They introduced T48F mutation in mSA and observed a modest 20–40% improvement in the binding kinetics[12]. Often structure-based protein engineering involves the application of various computational tools. Such advanced rational design is frequently used to understand protein-ligand interaction, define a 3D structure of an active site, calculate free energy and so on[13]. This method is applicable because X-ray crystallography, Nuclear Magnetic Resonance (NMR) spectrometry or other structural biology techniques allow the determination of protein structures at atomic resolutions and the implementation of molecular modeling to identify hot spots for mutagenesis. Generally, different computational tools have been used to predict hot spot residues of protein-protein interaction from the structure of unbound proteins[14]. Agrawal and co-workers presented an elite computational tool, termed Spatial Interaction Map (SIM) by which protein hot spot residues can be predicted with 36%-57% accuracy[15]. Furthermore, Tuncbag *et al*. presented a method to identify hot spots based on solvent accessibility, conservation and statistical pairwise residue potentials (pp) of the interface residues [16]. Both in Alanine Scanning Energetics Database (ASEdd) and in Binding Interface Database (BID), the predicted hot spots were found to match with the experimental hot spots with 70% accuracy.For detailed information about other available tools especially for protein-protein interactions analysis and hot spots residues prediction, an excellent review paper by Tuncbag *et al*.[17] is suggested. However, the ultimate goal of computational rational design is to design a 3D structure of an active site that may have desired catalytic activity. In certain protein design calculations, an experimentally determined high-resolution structure is used as a template to design new sequences. This strategy has led to the fortunate design of many novel and well-folded proteins. Most computational protein design studies have used crystal structures as templates[18] while NMR structures have used in a few cases. Computational protein design approaches are usually software based. In some cases, while NMR data was used together with these software, possibility of finding reliable results is reduced[19]. For this reason, most researchers still prefer X-ray crystal structures for computational protein design.

Compared to other well-established protein engineering methods such as simple rational design and directed evolution, computational approach allows much more mutations in sequence space. For example, more than a dozen of mutations can be introduced in a single step. There is no specific stepwise method of computational strategy for protein engineering. It depends on the particular interest of researchers, but in most cases, researchers used a computational approach to determine the specific hot spot residues for mutation.

An overview of the general procedure for computational rational design of enzymes is shown in Fig. 1(B). Last year, an excellent review by Hein *et al.* was published, where authors discussed various computational tools for enzyme catalysis engineering[20]. The authors claim that the principle elements of the computational design method are conformational sampling, energy functions, constraints, search algorithms and ranking. In the first stage, conformational sampling indicates a large number of independent point mutation sites[21] in the designed enzyme structure. Some software tools are involved in this step. For example, FOLDX[22] and ROSETTA[23] are used to test all possible combinations of mutations and to discover feasible combinations of more mutations. In the second stage, if multiple mutations are allowed at the same time, energy functions are required for special adaptations as the number of required energy calculations becomes extremely large. Then, the use of constraints is a simple tool to shun unwanted conformations, such as those that are incompatible with an active enzyme. After that, from the large sequence–conformational space, suitable sequence with lowest energy conformation can be found by algorithms search and then appropriate designs are selected by ranking.

Furthermore, Damborsky and Brezovsky grouped computational rational methods into three main categories: bioinformatics, molecular modelling, and *de novo* design to design and engineer enzymes[24]. Firstly, Bioinformatics tools involve various internet based software like ZEBRA[25], SWISS-Model[26], JANUS[27], ASRA[28] and 3DM[29] to analyze enzyme functional subfamilies, to homology modeling of protein 3D structure, to analyze multiple-sequence alignments and to know the underlying regularity of the protein property respectively. The choice of the computational tools depends on the predicted results. Secondly, in molecular modeling approaches, researchers use detailed structural information of the target protein to analyze its shape and size of the tunnel that enables substrate and product entry or release[30]. In order to change substrate specificity, enantioselectivity or regioselectivity of enzymes, several geometries-based software like MOLE[31], CAVER[32] and POREWALKER[33] have been developed. At last, the *de novo* protein design is acutting-edge protein engineering method by which tertiary structures of proteins can be predicted from the simple amino acid primary sequence. The *de novo* design has demonstrated significant progress in protein structure determination through computational techniques. Traditional *de novo* design referred to as single state design (SSD) which focuses primarily on the optimization of amino acid sequences for coordinates from a single protein backbone template. Davey and Chica found two reasons behind the recent progress of SSD[34]. The first one is the suitable protein backbone template coordinates for the desired function. The second is the simulation goals could be accomplished with the use of a design approach.The authors also demonstrate that in two cases, SSD could not be applied. The first limitation is the problem with protein design, when desired and undesired states are considered for designing. The second limitation arises when desired conformational or chemical states of a protein is multiple. In such cases, multistate design (MSD) should be applied.Sequential application of multiple computational methods and re-designing of proteins have gained momentum in structure-based engineering. Chen and co-workers employed multiple computational methods to redesign glutamate dehydrogenase (GDH), with the intention to reduce the ammonia flavor of natto which is a popular traditional Japanese soybean food obtained by fermentation using *Bacillus subtilis* natto[35]. GDH catalyzes the deamination of glutamate and produces alpha-ketoglutarate responsible for odorous flavor of natto.The researchers constructed a 3D model of GDH complexed with cofactor $NADP^+$ by eight threading programs, namely SAM, SPARKS, FUGUE, COMA, HHSEARCH, PROSPECT, SP3, MUSTER.Segment matching and *ab initio* modeling was employed as the computational approach to predict the aligned region structures and the unaligned region structures of the target proteins respectively. After substrate glutamate-structural model docking analysis, they observed Lys80, Lys116, Arg196, Thr200, and Ser351 residues in the active site are responsible for forming a hydrogen bonding network with the substrate. Substitution of these residues with other amino acids (K80A, K116Q and S351A) results in significant decrease in the substrate binding affinities.

Generally, hot spot residues are found mostly in the active site of enzymes. For this reason, traditionally it was thought that only mutations in the active site could improve enzyme properties. Nowadays, due to the great advances in computational tools, the residues outside of the active site can be picked up for mutagenesis with good improvement in enzyme properties. With the help of molecular dynamics and quantum mechanics, this new strategy has played a vital role in structure-based protein engineering and created a new field of research that will advance tremendously in the future. Moreover, Quantum mechanics are also playing a significant role in uncovering detailed reaction pathways by explicitly showing energetically favorable molecules and the magnitudes of energy[36]. This information regarding structures and energy levels simplifies computational rational design. Energetically unfavorable protein molecules can be easily detected by this technique. Then, the possible mutation site can be designed for desirable one, which is extremely complicated without quantum dynamic information. In addition, using molecular dynamics and quantum mechanics, Zheng and co-workers explored the correlation between the protein structure and catalytic efficiency of butyryl cholinesterase (BChE) mutants against (-)-cocaine by modeling the rate-determining transition state (TS1)

[37]. Interestingly, their results suggest that, instead of mutations in the active site, the mutations on certain nonactive-site residues can drastically improve the catalytic efficiency of the enzyme. On the other hand, by combining various computational methods, it is now possible to design artificial proteins that mimic native enzymes[38]. After all, recent advances in the structure-based protein design have been enhancing the use of computational tools in protein engineering. The application of forceful computational tools to functional protein design has produced many exciting results. Computational protein design is now considered as an attractive engineering strategy that can be overcome the bottlenecks of traditional methods. On the other hand, the most challenging step of this method is accurate modeling, which can limit the success rate of computational protein design. Other problems include low catalytic activity and high failure rates. However, in the near future, further improvement in algorithms, software tools and accurate calculation methods will overcome these obstacles.

STRUCTURE-BASED ENZYME ENGINEERING

Often native enzymes display hindrance properties for the industrial applications. High stability, solubility, catalytic ability, substrate and co-factor specificity are the most desirable industrial traits of enzymes. Structure-based engineering approaches are often exploited to obtain these properties. Examples of many engineered enzymes and their potential applications are summarized in table 2.

Enzyme stability

Enzymes stable to temperature, water and alkali are most desirable in industrial applications. Different protein engineering methods have been applied to improve enzyme properties among which structure-based engineering strategy is the accurate, specific and mostly applicable method. Some notable research works have been published recently. The α-amylase enzyme family is responsible for starch hydrolysis and broadly used in food, pharmaceutical and textile industries[39].A study by Deng and co-workers improves the thermostability of alkaline α-amylase from *Alkalimonas amylolytica* through structure-based rational design and systematic engineering of its catalytic domain[40].Swiss-Model was used to identify structural homologues and to predict structure. From 3D crystal structure analysis, the authors replaced histidine residues with leucine (H152L, H164L, H171L, H182L and H209L) to stabilize the least similar region in domain B. They also changed glycine, proline and glutamine residues in domain A to stabilize the highly conserved α-helices. After amino acid substitution, PoPMuSiC and Accelrys Discovery Studio algorithm were applied to predict the folding free energy change (ΔΔG),and to calculate the number of hydrogen bonds, salt bridges and aromatic–aromatic interactions respectively. Finally, the laboratory found 4 variants among 15-point mutants that show enhanced thermostability. However, in the first section, we mentioned that, the most crucial task for improving enzymes is the identification of critical sites for applying mutation. Several methods have been found to predict which sites/positions should be targeted for mutagenesis and can contribute to enzymes thermostability[41]. Different techniques have been applied in order to determine the residues responsible for low thermostability. A study by Wang *et al.* used multiple-sequence analysis (MSA) and molecular dynamic simulations (MDS) approaches to determine instable residue[42]. Using these approaches, they identified four residues (Valine (V), Glycine (G), Aspartic acid (D) and Serine (S)) in the active site expected to affect the thermostability of *Streptomyces sp*. strain S9 xylanase XynAS9. Five mutants (V81P, G82E, V81P/G82E, D185P/S186E, and V81P/G82E/D185P/S186E) were constructed by replacing these four residues with proline or glutamic acid and all mutants show improved thermal properties than wild types. In addition, Reetz and co-workers discovered a new approach to select the sites for amino acid exchanges[43]. They developed 'B-factor iterative test' (B-FIT) based on the observation that certain residues in protein crystal structures cannot be fully resolved due to a high flexibility of amino acids. The author suggested that, highest B factor amino acids should be replaced in order to increase thermostability of enzymes. Conversely, low B factors site in nonregular structural units should be focused when low thermostability is the desired property. At present, researchers widely use the program PyMol or the B-FIT software in identifying mutation site to increase thermostability[41, 44]. .Interestingly, not only amino acids positions, but also other factors like the collaborative effects of several forces such as disulfide bonds, hydrophobic interactions, hydrogen bonds and salt bridges are responsible for enzymes thermostability. The lid hinge region of *Rhizopus chinensis* lipase is the most flexible part of the structure. Researchers introduced a disulfide bridge between F95C and F214C in the hinge region of the lid for increased thermostability, presumably due to the stabilization of lid domain. They observed that the disulfide variant shows improved thermostability compared to the parental enzyme[45]. Likewise, Gall and co-workers improved thermostability of anesterase from *Bacillus subtilis* by domain exchange[46]. The research work strongly supports that modification outside of the active sites can also improve enzyme stability. Different computational algorithms, such as attribute weighting, have been applied to finding amino acid composition attributes that contribute to enzyme thermostability[47]. Due to the advance of these computational tools, it is now possible to predict enzyme thermostability before applying engineering techniques.

Substrate specificity

Structure-guided engineering strategies are also applied to improve enzyme specificity. Computational tools, crystal structure analysis and homology modeling technique have made significant progress towards this goal. Following these strategies, Midelfort and co-workers engineered *Vibrio fluvialis* aminotransferase (Vfat) with improved substrate specificity for the synthesis of imagabalin, an advanced candidate for generalized anxiety disorder[48].Based on the PDB structure of 1D7R and 2EO5, homology models of the *V. fluvialis* protein structure were created by the Accelrys Discovery Studio and Schrodinger Prime program. Mutations on wild type Vfatlead to an improved enzyme with a 60 fold increase in reaction velocity for transamination of (R)-ethyl 5-methyl 3-oxooctanoate to (3S,5R)-ethyl 3-amino-5-methyloctanoate. Moreover, natural products derivatization by engineered enzymes is now possible for the development of novel compounds with improved properties. For example, installing halogens onto natural products can result in improved quality compounds, which may be achieved by enzymatic halogenation. Glenn *et al*.engineered the flavin dependent halogenase RebH to allow chlorine onto tryptamine rather than native substrate tryptophan[49]. Then, they transformed the tryptamine-specific RebH into *Catharanthus roseus* and observed the halogenated alkaloid 12-chloro-19, 20-dihydroakuammicine. Likewise, combination of metabolic engineering strategies with the structure based enzyme engineering has expanded the intermediary metabolism of *E. coli* from C5 to C8 alcohol production, which is not readily produced by microorganisms. Because of the low water solubility and higher energy density, long carbon chain molecules (C>5) are attractive biofuel targets. Based on crystal structure and homology modeling, Zhang and co-workers designed the active sites of 2-ketoisovalerate decarboxylase (KivD) from *Lactococcuslactis* and 2-isopropylmalate synthase (LeuA) from *E. coli*.[50]. KivD was engineered for substrate specificity towards (S)-2-keto-4-methylhexanoate instead of its normal substrate 2-ketoisovalerate. And LeuA was engineered for increased activity towards (S)-2-keto-3-methylvalerate. The combination of KivD and LeuA mutants produce non-natural alcohols which are not produced in the wild type pathway.

Co-factor specificities

Enzymes use cofactor (NAD(P)(H), FMN(H), FAD(H), Mg2+, Zn2+, Fe2+) either for group-transfer reactions or as redox partners. In order to change the cofactor patterns of enzymes, structural enzyme engineering has been applied with important outcomes. The aldo-ketoreductase (AKR) superfamily, which uses nicotinamide as a cofactor, has a highly conserved cofactor binding pocket[51]. Most enzymes in this superfamily show high preference for NADPH than NADH. Amino acid substitutions in the cofactor binding pocket in a thermostable AKR from *Pyrococcusfuriosus*, AdhD, have resulted in improved catalytic specificity toward NADH[52]. Homology modeling was used to locate the interacting amino acids with the cofactor. Site-directed mutations were performed and a double mutant K249G/H255R was found with superior activity to NADH. Remarkably the double mutant enzyme does not lose its specificity with NADPH. Although the conversion of an NADPH - preferring enzyme to an NADH - preferring enzyme is desirable due to commercial reasons, it was difficult task in the past. 3D structure analysis and computational tools are making the task more accurate and easier than the past. Crystal structure analysis and the amino acid alignments of different NADH-dependent and NADPH-dependent enzymes can easily be done at this time. This analysis assists researchers to identify vital residues for enzyme-cofactor binding. After that the residues can be subjected to mutagenesis for finding desirable properties. The yeast *Candida tenuis* xylose reductase (CtXR) catalyzes the conversion of D-xylose into xylitol. CtXR can utilize NADPH or NADH as co-substrate in this conversion process, but the affinities of this enzyme towards NADPH or NADH is distinctive. Usually NADPH is 10 fold preferred than NADH. Inside host cells, changes in the cofactor specificity of CtXR towards NADH are expected to result in a global cellular response with positive effects on ethanol yield and productivity. Moreover, NADH is 10 to 20 fold more stable and 10 fold cheaper than NADPH. By structural and functional characterization of CtXR, Petschacherand co-workers found two different protein conformationsable to accommodate the presence and absence of the 2′ phosphate group of the co-substrate[53]. Subsequently, site-directed mutagenesis on NADPH interacting amino acids has resulted in mutants with improved NADH selectivity.

STRUCTURE BASED ANTIGEN AND ANTIBODY DESIGN

The traditional vaccine production involves identification of empirical antigens on the surface of pathogens. After that, selected antigens are isolated, inactivated or attenuated in order to avoid undesirable infections in the recipient body. Although the basic principle of vaccine production has not changed, however themodern methods involve the accumulation of experimental data and structural analysis of antigen-antibody complex. This provides us a better understanding of antigen recognition mechanism. The structure-based antigen design has emerged as a strategy for next-generation vaccine development. Its prerequisite is the detailed information about 3D structure of an antigenic protein, which provides atomic-level information on the overall fold and epitope location/formation. Different approaches using structural and computational biology have been applied frequently to identify epitopes [54]. Recently, Lassaux and co-workers presentedan approach integrating the structural and computational biology with

immunological tests for identifying epitopes in oligopeptide-binding protein A (OppA) antigen from *Burkholderia pseudomallei*[55]. OppA is part of the oligopeptide transport system that involved in nutrient uptake and recycling of cell-wall peptides. Various simulation tools, namely GROMACS 4.5.1 software package, GROMOS96 force field, and the SPC water model were used for epitope discovery in OppA. At last, three potential epitopes (COMP1–COMP3) were identified.

B-cell epitopes are antigenic residues that recognized and bound by antibodies produced from B cell[56]. Identification of the exact location of B-cell epitopes is crucial in antigen-antibody complex study [57]. There are two general approaches for B-cell epitope identification, structural and functional. Roggen reviewed currently available methods for predicting B-cell epitopes on proteins[58].Among various methods, structural method is the most accurate. It is based on the antigen-antibody complex structure determined by X-ray crystallography[59], electron microscopy (EM)[60]and NMR[61]. Computational prediction of conformational B-cell epitopes from antigen primary structures has gained significant popularity in vaccine improvement[62]. In a recent study, Gourlay and colleagues demonstrated that structure-based computational methods allow prediction of B cell epitopes, a crucial step for antigen selection and optimization in vaccine development[63]. Moreover, antigens or immunogens designing is also expected to elicit neutralizing antibodies (NAbs) for viruses, such as influenza[64] and HIV[65]. HIV-1 has been attracting much attention from researchers because of the lack of appropriate vaccines since last decade. A few portions in the HIV-1 envelope spike regions such as the CD4-binding site on gp120, gp120 co-receptor binding site and the gp41 MPER (membrane proximal external region) are structurally conserved among diverse isolates. These regions are the potential targets for antibody binding[66]. Structural analysis of these envelope glycoproteins strengths our understanding of envelope functions and it provides a blueprint for structure-based vaccine design[67]. Ofek and co-workers designed epitope scaffolds to elicit anti-HIV-1 antibodies through computational strategy[68]. They demonstrated the elicitation of structure-specific antibodies against the HIV-1 gp41 epitope by using the broadly neutralizing antibody 2F5.With Rosetta program, other researchers designed an epitope scaffold on the basis of the binding mode of 4E10 antibody that recognizes the MPER of gp41[65]. They observed that the scaffolds bind to 4E10 with higher affinities compared with only the MPER peptide epitope itself.

On the antibody side, with the advent of computational techniques, protein modeling and design has begun to play a significant role in improving antibody function. Using the structure-based approach, antibodies can be modified for improved stability[69], solubility[70], binding affinity[71] or altered binding specificity[72]. Utilization of crystal structural information greatly increases the chance of finding desired antibody. Following this strategy, Thakkar and co-workers used the anti- methamphetamine single chain fragments (scFv6H4) crystal structure to design point mutations for high-affinity antibody against methamphetamine (METH) and amphetamine (AMP)[73]. They found a mutant (scFv-S93T) with 3.1 fold enhancement in affinity for METH and 26 fold for AMP. Likewise, Kiyoshi and his groups improved the binding affinity of the mature antibody 11K2 towards its cognate antigen MCP-1 (monocyte chemotactic protein-1), an important therapeutic target in inflammatory diseases[74]. By combining *in silico* calculations and thermodynamics analysis, they observed that, only single-mutations carrying charged residues at specific location enhance the binding energy with the antigen. Moreover, *in silico* modeling accelerates the development of antibodies with improved properties[71]. Determination of critical residues responsible for binding site is extremely complicated, particularly in the absence of structural information. In contrast, when antibody or antibody-antigen complex structures are available, residue detection is straight-forward[75].

Recently, structure-guided approach has shown a trend to engineer specific fragments of antibody such as Fab[76], Fv[77] and scFv[78]. Engineering of these fragments can generate soluble monomeric antibody with specific functions[79]. Moreover, often it can overcome poor tissue penetration problem found in clinically approve full size antibody[80].Using homology modeling, Miklos and co-workers designed a single-chain variable fragment for enhancing resistance to thermal inactivation[81].They used Rosetta computational design package to predict energetically favorable charged surface residues. Meanwhile, Sivasubramanian and colleagues reported a protocol for the development and implementation of Rosetta Antibody[82]. They combined comparative modeling of canonical complementarity determining region (CDR) loop conformations and *de novo* loop modeling of CDR H3 conformation. This modeling method is based on information with simultaneous optimization of VL-VH rigid-body orientation, CDR backbone and side-chain conformations. Although they observed a moderate-to-high accuracy docking prediction, modeling antibody structures for sequences with long H3 loops is still challenging.

In addition, structure-based approaches have been explored recent years to enhance receptor affinity [83].Following the approach, Han and co-workers performed a structure-based design of a repeat protein with high binding affinity for a target protein[84]. Based on the TLR4-MD2 crystal structure complex, researchers determined single amino acid substitution site and obtained variants with high binding affinity

compared to the wild-type decoy receptor. Likewise, the efforts to enhance the binding affinity of T-cell receptors (TCRs) through structure-guided engineering have been reported[85].Haidar and colleagues designed a human TCR to improve its peptide-MHC binding ability[86].For this purpose, they used CONGEN and Rosetta programs for structural modeling of point mutations. A protocol named ZAFFI was designed to combine the structural modeling of Rosetta with a novel scoring function that includes shape complementarity and desolvation terms. This protocol was used successfully to predict new mutants with improved binding affinity. In summary, computational and structural strategies can be applied to aid the development of biological therapeutics, such as antigens, antibodies and receptors. In the near future, the advances in various structure-based strategies could be significant driving forces for the progress in the pharmaceutical industry.

Table-1: Comparative advantages and disadvantages of different protein engineering methods.

Method	Advantages	Disadvantages	References
Rational design	Directed; feasible for engineering secondary structure and domain; high efficiency	Structure based method; structure-function interaction of target enzyme should be clear	[10],[87],[88]
Computational approach	Possible to apply up to 20 point mutation in single step; allow sequence insertions and secondary structure modification	Low catalytic rates; complexity in accurate modeling	[89],[90],[91],[92]
Directed evolution	Structure not require; mimic natural evolution	unfeasible to engineer domain and secondary structure; random	[87],[93],[94]

Table-2: Improving the properties of enzymes through structure-based engineering techniques and their applications

Enzyme	Microorganism	Target site for modification	Method	Improved property	Application	Reference
Xylose isomerase	*Actinoplanesmis souriensis*	Active site	Site-directed mutagenesis	Substrate specificity	High fructose corn syrup production	[95]
PQQ glucose dehydrogenase (PQQGDH-B)	-	Active site	Site-directed mutagenesis	Substrate specificity	Sugar oxidation	[96]
N-acetylneuraminic acid lyase (NAL)	-	Active site residues	Saturation mutagenesis	Substrate specificity	Influenza drug	[97]
Aniline dioxygenase (AtdA)	*Acinetobacter sp.*	Substrate-binding pocket residues	Saturation mutagenesis	Substrate specificity	Pharmaceutical industry	[98]
Lipase (LIP4)	*Candida rugosa*	Substrate binding site	Site-specific saturation mutagenesis	Substrate specificity	Production of fatty acids	[99]
Aspartyl-tRNAsynthetase (AspRS)	*Escherichia coli*	Active site residues,	Site-directed mutagenesis	Probing the electrostatic interactions	Genetic code translation	[100]
Pyranose 2-oxidase	*Trametes multicolor*	Active site loop	Saturation mutagenesis	Substrate specificity	Carbohydrate transformations	[101]
Xylanase	*Aspergillusniger*	Serine-threonine surface in active site	Site-directed mutagenesis	Improve thermo stability	Pulp bleaching	[102]
Alpha-amylase	*Bacillus megaterium*	Calcium-binding site	Site-directed mutagenesis	Thermal stability and calcium independenc	Starch-liquefaction process	[103]

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Methyl parathion hydrolase	*Ochrobactrum sp.*	C-terminal residues	Site-directed mutagenesis	Enhanced thermo stability	Agricultural pesticide	[104]
TL xylanase (TLX)	*Thermomycesla nuginosus*	N-terminal region	PCR primers	Increase thermo stability	Pulp bleaching and baking	[105]
Strictosidinesyn-thase	*Rauvolfia serpentina*	Active site residues	Site-directed mutagenesis	Substrate specificity	Alkaloid biosynthesis	[106]
Cyclodextringluca notransferase	*Bacillus stearothermophi lus*	Phe 191 andPhe 255	Site-directed mutagenesis	Cyclizing activity modulation	Bread industry	[107]
Endo-β-1,4-xylanase	*Bacillus subtilis*	Asp4 and Asp11 or His149	Rational design	Improve acid stability	Hemicellulos e degradation	[108]
Lipase	*Bacillus pumilus*	α-helix 1	Site-directed mutagenesis	Improve thermo stability	Chemical, food, leather and detergent industries	[109]
Glycerol dehydratase	*Klebsiellapneu moniae*	α-Phe60 and α-tyr525	Site-mutagenesis	Enhance pH stability	Polyestersynt hesis	[110]
D-glucose 1-dehydrogenase isozyme -IV	*Bacillus megaterium*	C-terminal region	Site-directed mutagenesis	Reduce substrate specificity	Blood glucose levels measurement	[111]
Endoglucanase	*Thermoascusau rantiacus*	Fhe16S and Tyr 95	Site-directed mutagenesis	Increase hydrolytic activity	Bioethanol production	[112]

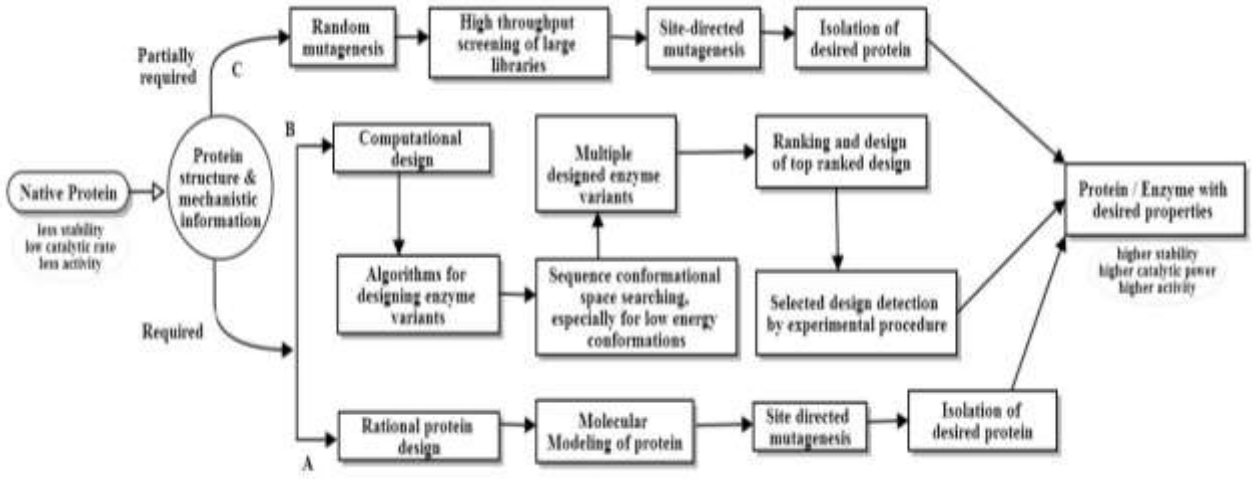

Fig-1: Schematic representation of structure-based protein engineering techniques.(A) Simple rational design; (B) Computational rational design; (C) Semi-rational approach.

CONCLUSIONS

With the rapidly increasing number of protein 3D structures available in PDB databases and the improvement of protein modeling tools, structure-based protein design has become an efficient and broadly applicable method. As a structure-based method, computer-assisted rational design has been considered as a straightforward route to alter protein properties and bridge the gap between rational design and laboratory evolution. This trend is boosted by the continuous improvement in the computational tools. The structure-based protein engineering is used to improve the enzyme activities, stability as well as substrate/co-factor specificities. In addition, computational epitope predictions/design using crystal structure as a template provides significant impetus to vaccine development. Structural information and newly developed computational tools are instrumental in improving antibody properties and better understanding of interacting residues in antigen-antibody complex. In the

future, the structure-based protein engineering will see more and more application in protein-related research fields.

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