An effective computational-screening strategy for simultaneously improving both catalytic activity and thermostability of α-L-rhamnosidase

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Abstract

Catalytic efficiency and thermostability are the two most important characteristics of enzymes. However, it is always tough to improve both catalytic efficiency and thermostability of enzymes simultaneously. In the present study, a computational strategy with double-screening steps was proposed to simultaneously improve both catalysis efficiency and thermostability of enzymes; and a fungal ∝-L-rhamnosidase was used to validate the strategy. As the result, by molecular docking and sequence alignment analysis within the binding pocket, seven mutant candidates were predicted with better catalytic efficiency. By energy variety analysis, three among the seven mutant candidates were predict with better thermostability. The expression and characterization results showed the mutant D525N had significant improvements in both enzyme activity and thermostability. Molecular dynamics simulations indicated that the mutations located within the 5 Å range of the catalytic domain, which could improve RMSD, electrostatic, Van der Waal interaction and polar salvation values, and formed water bridge between the substrate and the enzyme. The study indicated that the computational strategy based on the binding energy, conservation degree and mutation energy analyses was effective to develop enzymes with better catalysis and thermostability, providing practical approach for developing industrial enzymes.

1. Introduction

Natural enzymes usually only evolve relevant catalytic performance according to their own needs. When enzymes are used in industry, methods are needed to be explored to tailor their activity towards industrially relevant substrates, and these enzymes should also be optimized towards industrial reaction conditions.[1] To improve the production in industry which involves biocatalysts, increasing activity of enzymes for specific substrates is the key.[2]Besides, given that high temperatures in industrial processes including reaction, purification, packaging etc. provide benefits such as increased substrate solubility, improved diffusivity, decreased viscosity of the medium, and a lower risk of microbial contamination, thermostability of enzymes is another important property.[3, 4]

Enzymes obtained from natural recruitment and protein engineering have greatly contributed in various sets of applications. Over recent decades, the newly developed methods in the protein engineering, including directed evolution, semi-rational design, and *de novo*design, have enabled to obtain numerous better enzymes for the industrial application.[5-7] The catalytic activity and thermostability of many enzymes has been improved by directed evolution. By screening the triple mutant C168T/Q192H/Y7L with error-prone PCR and site-saturation, the thermostability and enzyme activity of GH11 xylanase from *Aspergillus fumigatus*

RT-1 were improved.[8] Lin et al. identified a N255D mutant by random mutagenesis with 14-fold higher activity than the wild type Horseradish Peroxidase.[9] Yin et al. also constructed a mutation library with error-prone PCR. In this library, three five-linked mutants Bgl1D2, Bgl1D6 and Bgl1D20 stands out to have 2.3-2.6 times higher hydrolytic activity, while only Bgl1D2 becomes more stable. It has seven times higher thermostability, whereas Bgl1D6, Bgl1D20 shows no significant change in thermostability compared with the wild type.[10]

Despite of the success of the above trials, directed evolution is unfavorable when one considers the experimental resources it requires for mutants in a large library. Semi-rational design of proteins deals with this problem by introducing bioinformatics to rationally reduce the size of the mutant's library. At present, the catalytic activity and thermostability of enzyme are mainly improved by semi-rational design. Generally, the non-conservative amino acids around the active site may be related to the catalytic performance of the enzyme, and hence the catalytic activity of enzymes can be improved by changing the non-conservative amino acids.[11] Moreover, based on the correlation between thermostability of proteins and factors such as hydrophobicity, packing density,[12, 13] number of disulfide bonds,[14] strength of electrostatic interactions,[15, 16] length of surface loops,[13] conformational rigidity,[17, 18] amino acid coupling patterns,[19] and local structural entrop,[20] bioinformatics software is generally developed to design proteins with good thermostability using proline theory,[21] B-fitter,[18]Rosetta,[22] molecular dynamics simulations[23] and disulfide by design et al[24].

Notably, among these semi-rational methods, mutants which have been designed to show improved thermostability, all display lower enzyme activity,[25, 26] and vice versa.[11] It is reasonable when we noticed the contradiction in the adjustment of protein structures between the two design strategies. Specifically, high catalytic activity was often obtained by reducing their surface hydrophobicity and hence increasing the flexibility of the structure,[27-29] while enzymes with good stability were designed by making the structure more rigid, which involves enhancing their surface hydrophobicity.[30-31] Thus, it is impossible to simply combine these strategies to design enzymes with both properties improved. Given that the high catalytic activity and good thermostability are both related to reduced costs, and vice versa, when one considers to optimize the total costs, which is often the case in the industry, new effective semi-rational design method is needed to be developed to simultaneously improve the catalytic activity and thermostability of enzymes.

To fill this need, here we proposed a double-screening strategy to obtain mutants with both properties improved based on compactional analysis and prediction of enzyme properties. Firstly, given that the non-conservative amino acids around the active site is related to the catalytic performance of the enzyme, mutation on the non-conserved residues in the catalytic region could bring potential higher activity. These mutants could be further screened virtually to select mutants with favorable heat stability. In this way, a relatively small library of mutants of potential simultaneous higher activity and thermostability could be further constructed and tested experimentally. Thus, not only does this strategy overcome the disadvantages of directed evolution, i.e. too large a library to do experiments, but also consider the shortcoming of the current semi-rational methods, i.e. limit the design too much on the local structure without considering the global effect. We tested this strategy on an extensively investigated enzyme in our lab, α-L-rhamnosidase.

α-L-Rhamnosidase is a glycoside hydrolase, which can effectively hydrolyze the rhamnose group at the end of most glycosides. It is widely used in the debittering of citrus juices,[32, 33]improving the aroma components of beverages.[34, 35] To date, only 29 α-L-rhamnosidases have been biochemically characterized, and six of them, namely *Bs* RhaB (PDB entry 2OKX), *Bt* 1001 (PDB entry 3CIH), *Sa* Rha78A (PDB entry 3W5M), *Ko* Rha (PDB entry 4XHC), *At* Rha (PDB entry 6GSZ), and *Dt* Rha (PDB entry 6I60) have been illustrated in crystal structures in GH78 family.[36-40] In previous studies, we cloned and expressed α-L-rhamnosidase (Rha1) from *Aspergillus niger*JMU-TS528,[41] which belongs to the GH78 family in the CAZy database. Simultaneously, we applied semi-conservative site-directed mutagenesis on the catalytic domain to increase the enzyme activity of Rha1.[42] We also used two different methods to improve thermostability of Rha1, PoPMuSiC algorithm and lysine-arginine mutation on the surface of rRha1.[43, 44] In this study, we applied the strategy mentioned above to improve the catalysis efficiency and thermostability simultaneously. Specifically, we computationally predicted the mutation in the catalytic region of Rha1 with the potential to improve the catalysis efficiency

and thermostability by the aid of molecular docking and conservation degree and energy variation analysis. The predicted mutants were then expressed and validated in the enzymatic activity and thermostability. This is the first semi-rational design to improve catalysis efficiency and thermostability simultaneously of enzymes, which could be helpful to effective design α-L-rhamnosidases and other important enzymes in the industry.

2. Results and discussion

2.1 Compactional screening the mutant candidates with better catalysis efficiency and thermostability

α-L-rhamnosidases, a glycoside hydrolase, exhibit a low sequence identity at only 20%–30%, but share a similar (α/α)6-barrel catalysis domain and several β-sandwiches.[36-40] Six crystal structures (PDB: 2OKX, 3W5M, 3CIH, 4XHC, 6GSZ and 6I60) from the GH78 family have been determined so far. By homology modelling with the crystal structures of α-L-rhamnosidases 2OKX, 3W5M, and 4XHC as templates, the structure of r-Rha1 was built and used for our research. In order to find out how the non-active-site residues of α-L-rhamnosidase contribute to the catalytic efficiency, bioinformatics analysis was performed. Specifically, docking between r-Rha1 and its substrate *p* NPR were performed and the interaction between them could offer a guide for site-directed mutagenesis. For example, Lu et al.[45] have reported that some amino acids identified from protein docking play key roles in catalysis. Bernardi has reported that hydrogen bonds involved amino acids around the catalytic domain contribute most to the contact between enzyme and substrate, which could be mutated to adjust enzymatic activity.[46] In the binding mode of r-Rha1 and substrate *p* NPR (Fig.1A),9 residues (Trp^{253}, Tyr^{293}, Thr^{301}, Val^{302}, Ser^{303}, Ala^{355}, Ser^{356}, Asp^{525}, Trp^{640}) of r-Rha1 were located around *p* NPR (Fig, 1B). Notably, some of these residues could be significant for enzyme activity and should not be mutated. According to structure of bound L-rhamnose from Fujimoto[37], which was sandwiched between two aromatic residues, Trp^{640} and Trp^{747}, the corresponding residues in r-Rha1, Trp^{253} and Trp^{640}, are such essential residues and should not be modified in site-directed mutagenesis. An amino acid sequence alignment of the available α-L-rhamnosidases from different sources revealed that, r-Rha1 shared a low similarity with other α-L-rhamnosidases (Figure S1), but the residues in the catalytic domains are well conserved (Table 1). The model of r-Rha1 exhibited that the side chains of Tyr^{293} and Trp^{253} were located near the substrate binding loop which were important in the binding of the substrate. In the predicted catalytic site, the aromatic rings of Tyr^{293} and Trp^{253} were parallel to the ring of rhamnose and presumably play roles in fixing the substrate through the pi-pi stacking interaction, which is consistent with what Xu et al [47] have showed, i.e. hydrophobic residues were located around the catalytic pocket. As a consequence, Trp^{253}, Tyr^{293} and Trp^{640} should be excluded for mutagenesis. Thus, the rest residues identified from the molecule docking, Thr^{301}, Val^{302}, Ser^{303}, Ala^{355}, Ser^{356}, Asp^{525}, were selected for mutation to test the effect on its catalytic activity. Particularly, 14 mutations, T301S, T301Q, T301G, V302S, V302A, V302N, S303V, S303G, A355N, A355G, S356I, S356Y, D525N, D525G were designed, according results of sequence alignment (Table 1).

To filter out mutants with high thermostability, the thermostability of 14 mutants were evaluated by mutation energy (stable) module of Discovery studio 2019 at 60, 65, 70, 75, 80 ^{o}C, and the calculation results were shown in Fig 1C. As a result, seven mutants were found to be stable at 60, 65, 70, 75, 80^{o}C. They are D525N, S356Y, D525G, S356I, A355N, S303V, V302N, with the stability from high to low in sequence. While the structure of mutant T301Q, T302S, T301S, V302A, S303G, A355G, T301G were unstable at different temperatures, it means the seven mutants have lower thermostability (see Table S2 for detailed data). Therefore, we obtained seven mutants D525N, S356Y, D525G, S356I, A355N, S303V, V302N through two rounds of screening, which may have both high enzyme activity and good thermostability, and could be verified experimentally.

2.2 Expression and characterization of mutant candidates with better catalysis efficiency and thermostability

The seven mutated enzymes were obtained by site-directed mutagenesis and further expressed in *Pichia*

pastoris. Similar to the wild type (recombinant Rha1, named as r-Rha1), the purified mutant enzymes showed a molecular mass of approximately 100 kDa (Fig. S2). Fig. 2A showed the relative hydrolytic activities of the mutants compared with the wild type r-Rha1. The mutants V302N, S303V, S356I and D525G show lower hydrolytic activities compared to r-Rha1. On the contrary, the hydrolytic activities of A355N, S356Y and D525N were noticeably enhanced by 45%, 80% and 180% than that of r-Rha1, respectively. To further investigate the effects of A355N, S356Y and D525N on the catalytic machinery of enzyme, the kinetic behaviors of A355N, S356Y and D525N on*p* NPR were evaluated. As shown in Table 2, bothK_{m} and k_{cat} values of A355N, S356Y and D525N were lower than the values of r-Rha1, whereas$k_{\mathrm{cat}}/K_{\mathrm{m}}$ values of all three mutants were increased. The mutant D525N showed the highest hydrolytic activity and catalytic efficiency among the mutants and r-Rha1. Specifically, D525N increased 2.85-fold of hydrolytic activity, and 1.54-fold of $k_{\mathrm{cat}}/K_{\mathrm{m}}$value compared to the wild type r-Rha1.

To gain a deeper insight of the mutagenesis effects of A355N, S356Y and D525N on the characteristics of enzyme, the optimum temperatures and thermostabilities were examined. Similar to wild type r-Rha1, the optimum temperatures of three mutants were 60$^{\mathrm{o}}$C (Fig. 2A). While the mutants A355N and S356Y showed markedly weaker thermostabilities than r-Rha1 (Fig. 2C), mutant D525N exhibited slightly stronger thermostability than r-Rha1. When the thermostabilities of r-Rha1 and its mutants were tested at 60$^{\mathrm{o}}$C, 65$^{\mathrm{o}}$C and 70$^{\mathrm{o}}$C, the half-lives of D525Nare shown to be 42 min, 2 min and 1 min longer ($T_{1/2}$) than r-Rha1(Fig. 2B(b-d)), respectively. Thus, D525N was identified to have both improved thermostability as well as increased enzyme activity, which demonstrates our screening method is helpful in identifying mutants both improved enzyme activity and favorable thermostability.

2.3 Secondary structural characteristics of mutants with better catalysis efficiency and thermostability

In order to further explore the reasons why mutants A355N, S356Y and D525N with improved enzyme activity have two different characterizations for thermostability, far-UV CD was used to investigate the effect of site-directed mutations on protein structure. The result showed that the CD spectra of both WT and mutants were nearly 100% superimposed (Table S2). As showed in Fig. S3, both r-Rha1 and three mutants presented a negative peak at 205-245 nm a typical feature for α-helices, where the strong positive peak at 185-195 nm represented a typical signature of β-sheets. The secondary structure of r-Rha1 and the mutants A355N, S356Y and D525N were calculated based on the CD spectra. Slight decreases of α-helix and β-turn were observed in the three mutants, whereas β-sheet and random were increased. Specifically, the content of α-helix of WT, A355N, S356Y and D525N was 27.5%, 25.3%, 25.3% and 25.7% while the content of β-sheet was 26.6%, 27.6%, 29.7% and 28.6%, respectively. Meanwhile, the β-turn component decreased by 0.7%, 0.4% and 0.6% in mutants A355N, S356Y and D525N, respectively. In Fig. S4, theT_m value of the mutant A355N was almost unchanged compared to the wild type: Tm of S356Y was 0.73 times that of wild type while D525N was 1.1 times. This is consistent with what Mohd et al[48] have showed, i.e. the substitutions of one or a few amino acids, which lead to the improvements in thermostability and optimum temperature of the mutants did not cause a substantial change in the secondary structures of the enzymes. This means that the change of a single amino acid does not cause major changes in the secondary structure, and the difference of thermostability among these three mutants could not be attributed to the change of the secondary structure.

2.4 Computational analyses of inner structure and molecular interaction of mutants with better catalysis efficiency and thermostability

2.5.1 MD and conformational stability analysis

Based on the aforementioned experimental analyses of A355N, S356Y and D525N, MD simulations were performed on these three mutants to analyze their structural changes. The root mean squared deviation (RMSD) is the essential parameters to validate the quality of MD simulation. In the present study, the stability of wild type r-Rha1 and its mutants were determined by the deviations produced during the course of MD simulations. As illustrated in Fig. S5, r-Rha1 reached equilibrium after 4 ns of MD simulation, with a

RMSD value of 0.5 Å, while A355N reached equilibrium after 11 ns, with a RMSD value of about 0.8 Å, and S356Y and D525N reached equilibrium after 5 ns, with a RMSD value of about 0.7 Å. The RMSD is used for measuring the differences between the backbones of a protein from its initial structural conformation to its final position.[46] The higher RMSD values of three mutants than that of r-Rha1 suggested that mutants A355, S356Y and D525N can influence the backbone stability of the enzyme. A similar observation was reported earlier for several systems, including glycoside hydrolases and other enzymes. [49, 50]

2.5.2 Binding free energy analysis

To gain more insights of the effects of point mutations on the interactions between α-L-rhamnosidase and substrate, the binding free energies and individual energy components were calculated by using MM-GBSA method. The results showed that A355N, S356Y and D525N mutations enhanced the binding affinity between α-L-rhamnosidase and substrate, which were consistent with our experimental results. Comparing the individual components contributing to the binding free energy (Table 3), it can be concluded that the electrostatic and Van der Waal interaction contributed the most in the changes of the binding strength of A355N and S356Y, while in the case of D525N, besides electrostatic and Van der Waal interaction, polar salvation also plays a significant role. Overall, MM/GBSA binding free-energy analyses corroborated perfectly with the outcome of molecular docking and dynamics analyses, and revealed paramountly lower binding and energy for optimum activity temperature which indicated stably mutate enzyme substrate complex.[51, 52]

2.5.3 Interaction analysis

Interaction analysis of the conformations in the simulations of wild type and the mutant shows that when the alanine at position 355 was mutated to asparagine (Fig.3A and 3B), the amino acid side chain of asparagine was extended. Due to the effect of steric hindrance, Ser^{356} was pushed forward. Moreover, water bridge was formed between substrate *p* NPR and Ser^{356}, which strengthens the hydrogen bond interaction between the enzyme and the substrate, thereby increasing the activity of the enzyme on the substrate. Thus, these results were in conformity with the results of the docking MD simulation studies of previous reports, in which the formation of hydrogen bonds increases the activity of the enzyme on the substrate.[46, 52] When the serine at position 356 was mutated to tyrosine (Fig.3C and 3D), a π-π stacking was formed between the phenyl of tyrosine and the nitrophenyl ring of the substrate*p* NPR, which strengthens the interaction force between the enzyme and the substrate, and bring out improved enzyme activity. The correlation between the pi-pi stacking and the strength of the substrate-enzyme complexes is also seen in a coplanar mu-S bridged 1,10-phenanthrolinepalladium(II) dinuclear complex. [53]

As for mutant D525N, when aspartic acid at position 525 was mutated to asparagine, as shown in Figure 3E and 3F, i.e. the acidic negatively charged amino acid was mutated to a polar uncharged amino acid, the negative charge in the vicinity of Asn^{525} suddenly changed to positive charge. The strong change of local environmental charge led to a large change in the enzymatic properties, and could justify the increase of both enzyme activity and thermostability, as results from Naito et al.[54] that the change in the charge of the catalytic domain led to the increase of the activity and thermostability of the enzyme.

Thermostable enzymes with favorable catalytic efficiency are very limited in nature and hard to obtain. Therefore, how to effectively improve the thermal stability and catalytic efficiency of enzymes has always been a hot issue in research. In most literature reports,[28-31, 55] it is shown that the increased flexibility of the enzyme structure can improve the catalytic activity of the enzyme, and the increased rigidity of the enzyme structure can improve the thermostability of the enzyme. Based on the similar designing ideas, Sniha et al[28], Humer et al[25] and Ashraf et al[26] have demonstrate that it is impossible to use the corresponding rational or semi-rational design to achieve the goal of enzyme engineering, that is, to improve the catalytic activity and thermostability of the enzyme at the same time. Here, instead of adjusting the global rigidity, we selected mutation sites in the catalytic domain near the substrate binding, modifying only the local rigidity without changing the overall structure of the enzyme, and used a double screening strategy, successfully obtaining mutants with improved catalytic activity as well as thermostability. By using the semi-rational design procedure, the mutation at Asp^{525} , which is located within the range of 5 Å of the catalytic domain

bound to the substrate (Fig. 1A and 1B), were shown effective in simultaneous improvement of the catalysis activity and thermal stability. Th results indicate the new semi-rational strategy is very effective to improves both enzyme activity and thermostability. In addition, our study suggests that the 5 Å range around the substrate binding site is the effective candidate to conduct protein engineering mutation for improve enzyme activity and thermal stability.

Conclusion

In this study, we designed a computational strategy with double-screening step for the first time, with the attempt to develop enzymes with increased catalysis activity and thermostability. The fungal α-L-rhamnosidase was used to validate the strategy. First, through molecular docking and sequence alignment, seven mutant candidates, i.e., D525N, S356Y, D525G, S356I, A355N, S303V and V302N were predicted with improved catalysis efficiency. Furthermore, three of the seven mutant candidates were predicted with better thermostability by mutation energy (stable) analysis. By enzyme expression and characterization analysis, the mutant D525N among the three candidates was confirmed with improved catalysis efficiency and thermostability. Moreover, microstructure analysis in MD simulations revealed the mutation D525N was located within the range of 5 Å of the catalytic domain, improving RMSD, electrostatic, Van der Waal interaction and polar salvation values, and forming water bridge between the substrate and the enzyme. These results not only provide an effective strategy for developing excellent enzymes for industrial applications, not only add the theoretical basis for enzyme engineering.

3. Materials and Methods

3.1 Strains, plasmids, reagents and substrates

Escherichia coli strains DH5α were used for propagation and manipulation of plasmids, and *P. pastoris* SMD1168 was used for protein expression. The plasmids pPIC9K were used for gene cloning and expression, respectively. The tool enzymes of *Sal* I, T_4 DNA ligase, *Taq* DNA polymerase, DNA marker, and *d* NTP were obtained from Takara Biomedical Technology (Beijing) Co., Ltd. And the antibiotic ampicillin was obtained from Solarbio/Life Sciences (China) Co., Ltd. (Beijing, China). All primers were synthesized by Invitrogen/Life Technologies (Carlsbad, USA). The substrate 4-nitrophenyl-α-L-rhamnopyranoside (*p* NPR), was purchased from Sigma Co. Ltd. (St. Louis, MO, USA). All other reagents used in this study were of analytical grade.

3.2 Compactional screening the mutant candidates with better catalysis efficiency and thermostability

The structures of α-L-rhamnosidase Rha1 (GenBank code: AGN92963.1) and its mutants were obtained by homology modeling using Modeller 9.15. Energy minimization was carried out using Discovery Studio2019. The three-dimensional structure model of *p* NPR was contructed by Chem Bio Office 2017. The simulated protein structure was docked against*p* NPR using AutoDock4.2. In general, the docking parameters for AutoDock4.2 were kept as default values. [(145.704, 166.348, 34.581), (60 Å×60 Å×60 Å)] grid map was used in docking calculations. Finally, the best poses were analyzed and visualized using Discovery studio 2019.

3.3 δνστρυστιον οφ α-Λ-ρηαμνοσιδασε ρ-Ρηα1 μυταντς

Site-directed mutagenesis to replace the specific residue as identified above was achieved by the KOD-Plus-Mutagenesis Kit (Toyobo, Japan) using the specific primer pairs and the DH5α/19-9k plasmid as a template for polymerase chain reaction (PCR) [42]. Then the product of PCR was transformed into freshly prepared *E. coli*DH5α by thermal shock at 42^{o}C for 90 s. The cell suspension was cultivated at 37 °C in luria-bertani medium containing 100 μg/mL ampicillin.

The genes were transformed into *P. pastoris* SMD1168 cells for protein expression. *E. coli* transformants were pooled, and the plasmid DNA was extracted using a TIANprep Mini Plasmid Kit (Tiangen, Beijing, China). The resulting recombinant vectors were separately linearized with *Sal* I, and transformed into

P. pastoris SMD1168 using a Gene Pulser Apparatus (Bio-Rad, Hercules, USA). The transformants were scanned after growing the clones on minimal dextrose medium plates at 30^{o}C for 3 d. Transformed yeast cells were selected on yeast extract peptone dextrose agar-plates containing 2.5 mg/mL G418 (Transgen Biotech, China). Colony PCR was carried out to confirm that whether or not the α-L-rhamnosidase gene has integrated into the genome. The General (5'AOX, 3'AOX) and Specified primers (Q9K-F, Q9K-R) as shown Table S3 were used for the selection of clones.

3.4 Measurement of catalytic activity

Enzyme expression and purification of r-Rha1 mutants, the α-L-rhamnosidase activity follows the same protocol in the previous study.[56] For kinetic experiments, five different concentrations (within 2-6 mM) of *p* NPR were used to incubate the purified α-L-rhamnosidase at 60^{o}C and pH 4.0 (20 mM of citrate acid buffer) for 10 min. The K_{m} and V_{max} were calculated from Lineweaver-Burk plots.[57] These values were further used to calculate the k_{cat} and the catalytic efficiency.

3.5 Measurement ofthermostability

The optimal temperatures for wild type and mutants were determined by measuring the enzyme activities at different temperatures (ranging from 30 to 80 °C) in 20mM citrate acid buffer (pH 4.0) with *p* NPR as the substrate. The thermostability was estimated by measuring the ratio of the residual activity to the initial activity of the enzyme. Samples were diluted in 20mM buffer (pH 4.0) to a protein concentration of 0.7 mg/mL followed by incubating at 60 °C, 65 °C, and 70 °C, respectively. After heat treatment, the samples were cooled on ice immediately. Relative activity was estimated with original activity taken as 100 %.

3.6 Circular dichroism spectroscopy analysis

Circular dichroism (CD) measurements were recorded from 190 to 260 nm at 25°C with a Chirascan Circular Dichroism spectrometer (Applied Photophysics, UK) at a scan rate of 100 nm/min, 0.25 s of interval and 1nm of bandwidth for identifying secondary structure. CD measurements were recorded from 190 to 260 nm at 20-100°C to determination of heat distortion temperature ($\mathrm{T_m}$).

3.7 In-silico analyses

3.7.1 MD simulation

The MD simulation was performed using GROMACS 5.1.4 on in-house super computing platform as earlier implementing the GROMACS 53a6 force field.[58] The initial structure was solvated with a simple point-charge model of SPC water in a box. Na^{+}ions were added to neutralize the negative charges in the system. The system was then subjected to a steepest descent energy minimization to give the maximum force below 1000 KJ/mol/nm. After energy minimization, the position restraint simulation of 2000 steps (2fs each steep) was implemented under NVT (the constant Number of particles, Volume and Temperature) and NPT (the constant Number of particles, Pressure and Temperature) condition. In the end, 50ns MD simulation was conducted on each ensemble. The root means square deviation (RMSD) and the root mean square fluctuation (RMSF), which is an important index for evaluating the protein structure, were calculated using GROMACS rms and rmsf tools. Time dependent secondary structure was calculated using do_dssp tool. The Particle Mesh Ewald method was used calculated long-range electrostatic forces. The neighbor list was determined with the Grid method. The time step of the simulations was 2 fs, and the coordinates were saved for analysis every 2 ps. Postprocessing and analysis were performed using standard GROMACS tools and Visual Molecular Dynamics (VMD).

3.7.2 The Binding free energy calculation

The binding free energies (ΔG bind) between amino acid residues were calculated using the molecular mechanics Poisson Boltzmann surface area (MM/PBSA) method.[59] The binding free energy (ΔG binding) between residues was calculated using the MM-PBSA method from a10-20 ns MD simulation trajectory. The binding free energies computed by this method could be represented using the following equations:

Protein + Subtract -Complex

Therefore, the binding free energy for each complex is calculated using the following equation:

$$G_{\text{bind}} = G_{\text{complex}} - (G_{\text{protein}} + G_{\text{subtract}})$$

Where G_{bind} is the total binding free energy, G_{complex}, G_{protein},G_{subtract} are the energies for the complex, the protein (wild type and mutants), and the subtract (p NPR), respectively.

Conflicts of Interest: The authors have no conflicts of interest to declare.

Author contributions

Lijun Li and Hui Ni designed research, Wenjing Li and Zheyu Wu performed experiments; Lijun Li and Wenjing Li analyzed data, Jianye Gong performed computational experiments and analyzed, Lijun Li, Wenjing Li and Jianye Gong wrote the paper, Yanyan Xu, Zedong Jiang, Hui Ni and Yi-Sheng Chen modified the paper. All authors read and approved the final manuscript.

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