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Original Article

Computer Aided Design of a Luciferase Like Haloalkane Dehalogenase Enzyme by Homology Based Rational Protein Design (HRPD) Method

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Abstract

Rational protein design is an important aspect to introduce novel characters to the enzymes. In this report, we describe a procedure for in-silico design of a novel haloalkane dehalogenase protein that exhibits luciferase property, which can be potentially used in biosensor applications. The haloalkane dehalogenase have a close structural homology with a lucifearse was used as the starting structure for design. Amino acids that are frequently interacts with the chromophre (colentrizine) was analyzed by docking and molecular dynamics simulation. The amino acids Asp170, Lys192, Arg193, Lys259, and Lys261 were selected in the enzyme structure for substitution purpose to generate mutant enzyme. Following several selection strategies, it was observed that the protein substituted with Phe in all the positions is the best one which was further validated by a 10 ns molecular dynamics simulation. The designed protein is then analyzed for its substrate specificity towards 10 selected toxic haloalkane compounds. The result shows, that the designed protein has also improved the substrate specificity towards four toxic pollutants.

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Introduction

In-silico protein design is an important tool to study structure-function relationship, which is a challenging prospective. One of the potential areas in protein engineering is directed evolution, where target enzymes can be created without the knowledge of its 3-D structure. However, this method requires a robust high throughput screening system. But with increasing availability of 3D structures in the structural database, the execution of various rational and computational methods for design of desired enzymes resulted in notable success [1-3]. In current protein design process, both computational, experimental and hybrid approaches have been used with good success rates. By applying mutagenesis and rational design techniques, researchers have created enzymes with altered functionalities and increased stability [4]. In computational protein design, the foremost requirement (or challenge) is to solve the complexity of protein structures computationally, where the mutant sequence is fitted on a desired structure template using a rotamer library and applying suitable energy functions [5]. Little knowledge of the complex construction/functional relationship of enzymes have been shown as a major constraint in the instance of rational design process [6]. Despite this, many advances have been made in the field with several computational algorithms being developed to design proteins [7-9]. One of the interesting applications in the rational design prospective is the design of proteins that can be appropriately implemented in biosensor application as described by Hellinga *et al*., [10]. Adopting this approach in a study, the specificity of maltose binding protein was successfully changed to a zinc sensor where the zinc binding signal is transduced into a readily detectable fluorescence signal, implementing the ligand binding to a suitable reporter group [11]. Similarly, another application of rational design is manipulation in the allosteric site for generic biosensor design [12]. Many researchers have also attempted for the implementation of rational design in *Aequorea* green fluorescent protein with an objective to engineer its variants that can be applied in bio-recognition process. The family of fluorescent protein based biosensors includes a diverse array of designs that utilize various photophysical characteristics of the proteins [13-14]. Following *in-silico* design methods, a molecular dynamics simulation is an important method to test the stability of different designed proteins. This is one of the popular computational methods that computes time dependent behavior of proteins and provide detailed information in terms of fluctuations and conformational changes [15-17].

Design of a Renilla luciferase like protein would be very much interesting as the same can be utilized in biosensor application. In the of luciferase enzyme, it contains a chromophore coelenterazine also refered to as luciferin [18]. The mechanism of fluorescence in this enzyme is well-established where the oxidization of coelenterazine to coelenteramide evolving light is coupled with green fluorescent protein (GFP) [19-20]. Since luciferase enzyme exhibits a broad range of emitting frequencies of bioluminescence, it is used as an excellent tool for several bio-analytical applications. The applications include monitoring gene expression in both plant and animal cells

[21-22], ultrasensitive detection of ATP phosphatase activity in case of microbial contamination, DNA sequencing, a tool for monitoring in vivo protein folding and chaperonin activity, etc [23-25]. The objective of the present work is to computationally design a haloalkne dehalogenase enzyme that will have both luciferase as well as biodegradation activity. Ultimately, this can be used as a biosensor application for detecting and monitoring toxic haloalkane pollutants.

Materials and Methods

System architecture used in this work

The configuration of the PC used from performing computation studies is as follows; OS Windows XP, Dual Core, RAM 2 GB, 250 GB HD, 1.76 GZ and for docking and MD simulation: HPC WIPRO Super Nova, 1 TF, Intel Xeon Quad Core 2.66 GHZ, with 16 nodes.

Selection of suitable halo acid dehalogenase and luciferase

The structure of haloalkane dehalogenase (PDB ID 1EDE) was retrieved from protein data bank (www.rcsb.org). Luciferase enzyme homologue was searched in PDB repository using PSI-BLAST (*NCBI*).

The selected luciferase enzyme from PDB was subjected to a 3 nano second (ns) MD simulation using Gromacs software to check the residues binding to the chromophore. These residues were visualized by AcclerysDS Visualizer. The molecular docking of luciferase chromophore on the haloalkane dehalogenase protein template was performed using Autodock vina tool [26], followed by a five ns MD simulation in Gromacs to check the pattern in the residue interaction of haloalkane dehalogenase with the docked chromophore.

Design strategy

The protein design was considered by selecting suitable amino acids that involve interactions with the chromophore. The docking and 5 ns MD simulation result was analyzed carefully and all conserved interactions with residues were selected for mutation. Simultaneously, four other protein complexes with the same chromophore structures available in PDB were also analyzed for the hydrophobic aromatic amino acid percentage neighbor to the chromophore. Based on the above result, 5 different amino acid positions were selected and substituted using PyMol mutagenesis module (www.pymol.org). The mutants were designed in 4 different ways. In the first approach, the amino acids were mutated according to hydrophobic and aromatic amino acids, based on their comparable molecular weight; similarly other three mutants were generated by mutating all the selected positions into Phe, Tyr and Trp respectively. One of the main approaches in computational protein design is managing the side chain conformations (rotamers) in the changed structure, therefore SCWRL4 tool was also used.

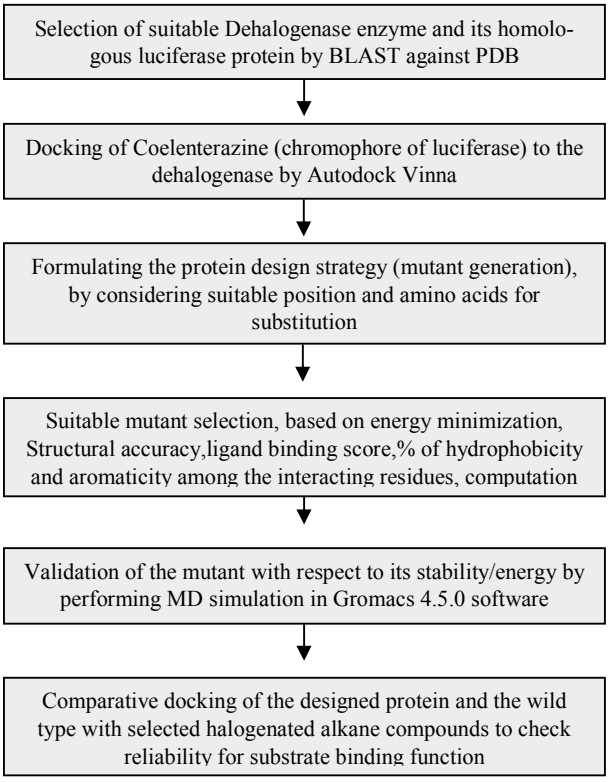
Selection of desired designed protein

All the mutant proteins, that are generated by the above mentioned methods were preliminarily checked for their number of alpha helix, beta sheet, turns using Rasmol. The numbers of salt bridges were computed using salt bridge prediction server (http://bioinformatica.isa.cnr.it/ESBRI/). Further, all the mutant structures were energy minimized by Spdviewer software followed by rotamer arrangement using Scwrl4 [27]. The Ramachandran plot and ERRAT value for the minimized model was computed using SAVES server (http://*nihserver.mbi.ucla.edu/SAVES/*). Along with above features the docking energy of chromophore to all mutants was also considered for selection of the desired structure. Finally MD simulation was performed at 10 ns in the final selected mutant; also many of the properties like stability and energy were computed.

Analysis of haloalkane binding affinity

The final selected designed haloalakne dehalogenase proteins that acquired the luciferase property were further analyzed for their affinity to the several haloalakane pollutant molecules. Marvin sketch was used to draw the chemical structures and energy minimization by the PRODRG server, with reduced charge option (http://davapc1.bioch.dundee.ac.uk/prodrg/).

After that, a molecular docking procedure was performed with the ligands and the unmutated haloalkane dehalogenase (1EDE.pdb) and the one designed by Autodock 4.2 tool [28]. Further, the binding affinities of the two proteins were compared. The methodology used in this work is represented schematically as follows:



Results and Discussion

The selected haloalkane dehalogenase (1EDE.pdb) from *Xanthobacter autotrophicus* was retrieved from the protein data bank. A PSI BLAST search in the PDB identified 2PSJ protein chain A (luciferase from *Renilla reniformis*) with an identity of 33%, E value of 4e -10 and query coverage of 60.5% to the query protein. Various parameters were computed for both the structures using Rasmol

and active site was obtained from Catalytic Site Atlas server (http://www.ebi.ac.uk/thorntonsrv/databases/CSA/). The results indicate that there is a considerable relationship between both proteins (Table 1) and as well share the hydrolase domain as established from the previous study. Therefore, the haloalkane dehalogenase (PDB ID 1EDE.pdb) was chosen as a suitable starting protein structure template for the proposed design purpose. For protein design purpose, the amino acids other than the homologous one (23-151) in the 1EDE protein were considered to ensure that these changes might not interfere with the structural stability. Hence coelenterazine interacting amino acids was chosen for substitution process. 2PSJ_A chain protein was carefully analyzed for neighbouring amino acids that interact with coelenterazine, the chromophore of lucifearse. In order to identify the interaction of preferable amino acids, an MD simulation was performed in water at 3 ns using Gromacs software and visualized (Table 2). From the results it was observed that the Phe residue in particular has good interaction with the chromophore of the luciferase protein.

Table 1. Observed features between 1 EDE and 2PSJ_A and alignment result as obtained from PSI-BLAST search.

No.	Features	1EDE	2PSJ_A
1	No. of Hydrogen bonds	210	211
2	No. of helix	18	18
3	No. of strand	11	10
4	No. of turn	35	33
5	Active site	Asp124, Asp266, His289	Asp120, Lys252, His285
6	Sequence length	310	319
7	Chromophore (as complex)	NIL	Coelenterazine

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1EDE   23 NYLDDLPGYPGLRAHYLDEGNSDAEDVFLCLHGEPTWSYLYRKMIPVFAESGARVIAPDF 82
          N LD Y H AE+ + LHG T SYL+R ++P E AR I PD

2PSJ_A 28 NVLDSFINYYDSEKH--------AENAVIFLHGNATSSYLWRHVVP-HIEPVARCIIPDL 78

1EDE   83 FGFGKSDKPVDEEDYTFEFHRNFLLALIERLDL-RNITLVVQDWGGFLGLTLPMADPSRF 141

             G GKS K + Y H +L A E L+L + I V DWG L R

2PSJ_A 79 IGMGKSGKSGN-GSYRLLDHYKYLTAWFELLNLPKKIIFVGHDWGAALAFHYAYEHQDRI 137

1EDE   142 KRLIIMNACL 151

             K ++ M + +

2PSJ_A 138 KAIVHMESVV 147
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Table 2. Amino acids interaction profile of 2PSJ_A protein that contains Coelenterazine after 3ns molecular dynamics simulation.

1 ns	2 ns	3 ns
Met174, Gly171, Leu195, Phe190, Asp162, Ile159, Pro157, Trp156, Phe181, Pro220, Glu155, Phe262, Ser263, Ile266, Glu144, Phe261, His285	Gly171, Phe180, Glu170, Leu165, Ile166, Ile163, Phe180, Asp162, Phe181, Pro220, Ile159, Val185, Pro157, Trp156, Glu155, His285, Phe262, Ser263, Glu144, Ile264	Gly171, Phe180, Phe181, Ile163, Asp162, Ile159, Pro157, Trp156, Glu155, Glu144, Ser263, Phe262, Phe261, His285

To confirm the Phe residue preference further, four other proteins that contain coelenterazine in complex were retrieved from the protein data bank (PDB ID 1S36, 2F8P, 4MRY, 4N1G) and visualized. The overall obtained results indicate that there is a strong affinity of Phe amino acids to the chromphore. molecular docking was performed between selected haloalkane dehalogenase protein (1EDE) with coelenterazine using Autodock Vina tool and the complex was subjected to a 5 ns MD simulation using Gromacs. The residues interacting with coelenterazine are tabulated in Table 3. The simulation result was carefully observed and the results indicate that in both proteins the residues that are part of the homologous region are not involve in the chromosphere interaction (Table 1, Table 2 and Table 3). Therefore, the residues Asp170, Lys192, Arg193, Lys259, Lys261 that are in close proximity to the most frequently interacting amino acids in haloalkane dehalogenase was chosen for mutation purpose. The above positions were chosen other than considering the most frequently interacting aromatic amino and hydrophobic amino acids, and also without disturbing the active site amino acid residues. As the substitution in these amino acids might change drastically in the overall social organization, function and stability of the protein. Four different mutant structures were prepared as described in the design strategy modules in the materials and methods section. The random design contains the substitution in the positions of 170 Asp/Leu, 192 Lys/Met, 193 Arg/Phe, Lys 259/Tyr, 261 Lys/Trp. Among the four designed protein, the substitution containing Phe in all the positions were found suitable, based on the computed features. The detailed features and positions are shown in the Table 4 and Figure 1.

Table 3. The residue interaction with coelenterazine after docking to haloalkane dehalogenase enzyme in 5 ns MD simulation.

1ns	2ns	3ns	4 ns	5 ns
Asp178, Trp175, Ala174, Gly171, Leu262, Leu263, Asp260, His289, Gly288, Ala195, Pro196	Ala174, Gly171, Leu262, Asp260, His289, Ala195, Pro196, Trp194, Trp175	Ala174, Trp175, Asp170, Gly171, Leu262, Gly288, His289, Trp194, Pro196, Ala195	Trp175, Ala174, Asp170, Gly171, Leu262, Asp260, Gly288, His289, Pro196, Ala195	Trp175, Ala174, Phe172, Asp170, Gly171, Leu262, Asp260, His289, Gly288, Trp194, Ala195, Pro196

Table 4. Feature selection for the designed proteins.

Mutant Types	Energy content (Kcal/mole)		Ramachand-ran plot (% of residues in favourable position)	Errat structure quality %	Ligand docking score from auto dock	% of hydrophobic + aromatic amino acids
	Before minimization	After minimization				
Random design	-1256.903	-15467.264	89.9	75.16	-7.5	77
All phe	-2266.106	-15080.509	89.9	96.46	-8.6	**77**
All trp	15628657.0	-13665.529	89.9	75.50	-7.4	62.5
All tyr	-5470.493	-15440.316	89.9	75.50	-7.8	76.9

The selected mutant protein (All_phe.pdb) was subjected to molecular dynamics simulated at 10 ns simulation by GROMACS using GROMOS96 43*a1* as force field. Standard protocol for simulation was used placing the initial protein structure in a cubic box filled with water, followed by energy minimization of the system without restraint using the steepest descent method for maximum up to 300 steps. The molecular dynamics simulation was set using

P-LINCS algorithm at 300 K temperature with a dielectric constant of 1 and a time step of 0.002 femto second (fs) for 5000000 steps under constant pressure during simulations. A similar protocol was followed in this work (excep change in time scale) for all MD simulation carried out throughout this work. To validate the suitability of designed protein in haloalkane pollutant degradation a molecular docking method was adopted. The structure of the pollutant molecules were obtained from pubchem database and processed by Marvin sketch and prodrg server. Further toxicity of the compounds was obtained from the information available at Toxnet server (http://toxnet.nlm.nih.gov). Ten molecules were selected for the docking purposes with the wild type and the designed haloalkane dehalogenase (all_phe) enzyme as receptors. The values in resolution indicate enhanced binding affinity of the designed protein towards 4 different haloakane pollutant molecules in comparison to the wild type (highlighted bold in Table 5). Besides the position, the conformation with respect to their predicted active site was obtained and presented in Figure 5.

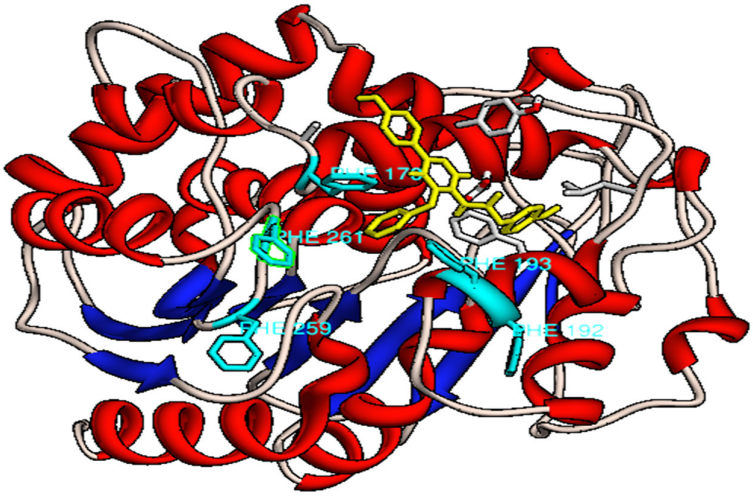

Figure 1. Showing docking of Coelenterazine (yellow colour) to the designed dehalogenase protein, the substituted position is shown in cyano colour.

Table 5. Binding energy profile to the wild type (1ede.pdb) and the designed one (all_phe) with the selected ligands along with their major toxic features.

No.	Name of the Haloalkane compound	Binding energy score (Kcal/mole) computation by Autodock 4.2		Toxicity prediction of the compounds by Toxnet data base
		With 1EDE.pdb	With all phe	
1	1,2,3 tribromopropane	-3.98	-4.14	Genotoxic
2	1,2 dichloropropane	-3.67	-4.06	Carcinogenic
3	1,2 dibromoethane	-3.97	-3.84	Gene mutagenic,birth defect
4	1,2 dibromopropane	-4.17	-4.59	Reproductive toxicity, osteogenesis inhibitor
5	1,2 dichloroethane	-3.28	-3.27	Embryo toxicity
6	1,3 dichloropropane	-3.66	-3.66	Developmental toxicity, Fish toxicity
7	1,3 di-iodopropane	-3.84	-4.41	Mutagenic
8	1 bromopropane	-3.62	-3.61	Neuroreproductive toxicity
9	1 chlorobutane	-3.74	-3.73	Water pollutant
10	1 chloropropane	-3.27	-3.26	Mutagenic

The successful execution of rationally designing a novel enzyme with the aid of computational methods is extremely commended. Various *in silico* protein design methods are available, which are suitable that take less time and are of low cost. The homology based rational design method we describe here can be considered as a good one. The basic principles involved in this study are by considering two facts. First being homologue, in which the diverse proteins with similar structures/domains, where in, if their sequence similarity is at least 30%, the protein is considered to be originated from a common ancestor. The origin of such a conserved architectural feature of the protein can be suitably implemented for rational manipulation of proteins as these features also account for maintaining the stability of the protein [29]. Many homology based approaches have been implemented successfully to design suitable proteins. Based on a modelled 3-D structure a number of mutants of a subtilisin-like protease are designed with the aim to increase its washing performance [30].

Also there is possible to introduce four key catalytic residues into NTF2 to create ascytalone dehydratase-like active site [31]. The other basis of design relies on the preference of amino acid interaction to the chromophore molecule that is essential for generating fluorescence. As in different type of fluorescent proteins a specific types of amino acid is found to be effective in signal generation that is applied in bio-monitoring purpose [32-33]. In the present case the structural homology among the two proteins and presence of common hydrolase domain has been proving their functional relationship. So, in the design strategy, we have introduced the suitable mutation, without disturbing the homologous portion of the protein structure and other essential residues. The major criteria that are used for validating the designed protein is computing the energy, and C-alpha backbone deviation (RMSD) representing the stability of the design protein. During the design process the residues were substituted after ensuring that no major change occurs in structure leading to instability (Figure 2 and Figure 3). Such stability parameters were confirmed from the comparative MD simulation in water.

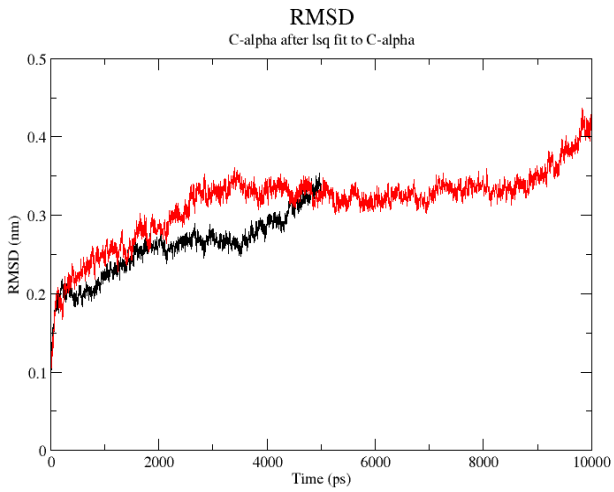

Figure 2. RMSD profile of Coelenterazine docked to designed protein (red) and wild type 1EDE.pdb (black) during 10 ns and 5 ns MD simulation, respectively.

The amino acid interaction analysis suggest that, the positions substituted Phe interacts frequently with the chromphore (Figure 4), that reflects the ability of light producing phenomena that of a typical luciferase protein. Since molecular docking is a confirmatory study to check the affinity of the ligands towards the protein receptor. In this work the docking process show an enhancement in affinity of some haloalkane pollutant molecules; hence the designed protein might be applicable for biochemical monitoring purpose.

However this is a computational work, which may be verified by the experimental approach for the same phenomena. The computational protein design approach has been coupled to the experimental methods for several ranges of applications.

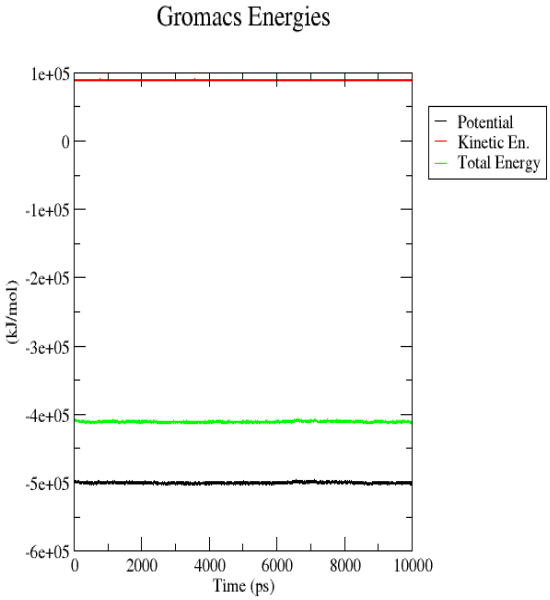

Figure 3. Energy profile of the designed protein with complex Coelenterazine during 10 ns MD simulation.

Conclusion

In the current work a computational design approach called as '*homology based rational protein design (HRPD)*' method was performed in haloalkane dehalogenase enzyme with an aim to reflect lucifease like property and not disturbing its original catalytic nature.Therefore, based on the 3-D structure analysis, sequence alignment, docking, amino acid substitution and MD simulation a luciferase like dehalogena was designed. The 5 positions Asp170, Lys192, Arg193, Lys259, Lys261 in haloalkane dehalogenase was found suitable when substituted with phenylalanine attained luciferase like property.

Additionally, docking with haloalkane pollutant showed enhanced affinity towards several halopropane pollutant derivatives indicating potential implication of this designed protein for biosensor purpose, in monitoring toxic haloalkane compounds like ,1,2,3 tribromopropane, 1,2 dibromopropane, 1,2 dichloropropane and 1,2 diiodopropane. The homology based protein design may be considered as one of the important method in the design strategy for rational design of other proteins for similar purpose.

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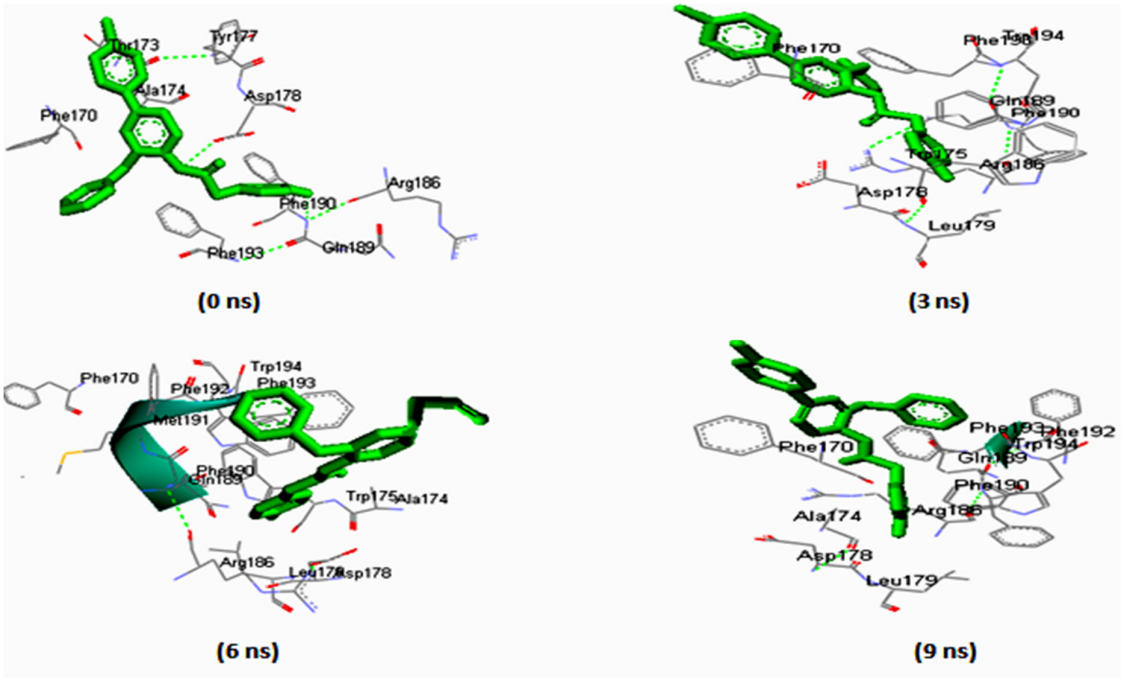

Figure 4. Snapshot of the substituted amino acids (grey colour) interaction to the chromophore (green colour stick) in the designed protein during the MD simulation at 10 ns.

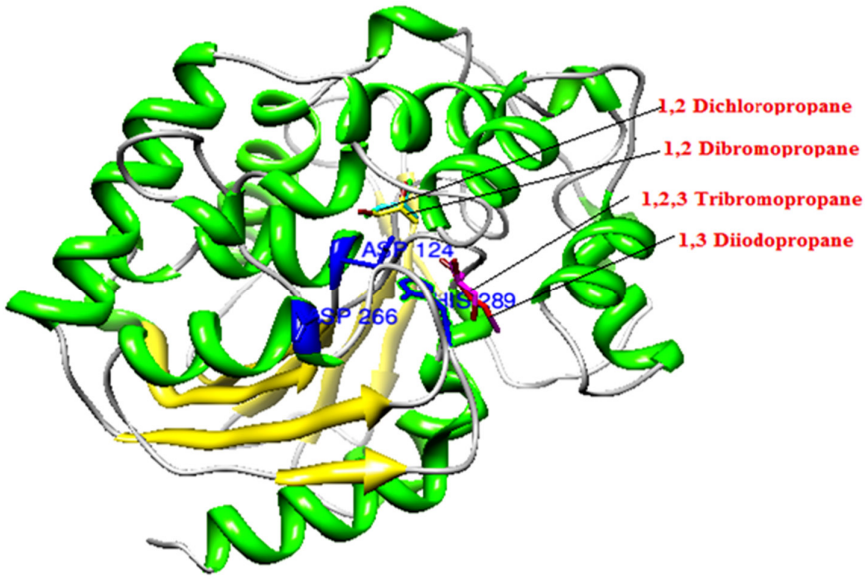

Figure 5. Docking conformations of 4 ligands to the designed protein with respect to predicted active site residues (blue colour).

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