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Hypothesis

An analysis of horseradish peroxidase enzyme for effluent treatment

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Abstract:
The present study explains computational methods to design thermostable horseradish peroxidase enzyme using the crystal structure available from Protein Data Bank (PDB ID: 6ATJ). Multiple mutations were introduced to the original enzyme and developed a model by using Modeler9.14. After designing the model functional effect was confirmed in terms of protein ligand binding by molecular docking using Autodock 4.2. The implementation of modeling steps is demonstrated in the context of performing mutations for particular amino acid residue on the ligand pocket of the horseradish peroxidase, to derive the desired ligand binding properties. The docking investigation of modelled HRP with Quercetindihydroxide using Autodock 4.2 software that six amino acid residues, P139, H42, A31, L174, A38, and G169 are involved in hydrogen bonding. More importantly, it provides insight into understanding and properly interpreting the data produced by these methods. The 3D model was docked with Quercetindihydroxide (a known horseradish modulator) to understand molecular interactions at the active site region.

Keywords: de-colourization, homology modeling, horseradish peroxidase, and industrial effluents.

Background:
Peroxidases are widely distributed among all living organisms in the nature. Linossier first coined the name peroxidase, after isolating it from pus cells **[1]**. Peroxidases can be extracted from plant **[2]** and animal cells, as well as tissues. Horseradish is a perennial herb cultivated mainly in temperate regions throughout the world. The roots of horseradish are a rich source of peroxidases. Peroxidase is a heme-containing oxidoreductase, which catalyzes the reductive cleavage of H_2O_2 by an electron donor. Commercially, peroxidase is used in diagnostic kits for immunoassays and also in treating various industrial effluents in bioremediation **[3]**. Quercetindi hydrogen consumption shows decreased incidence of neoplastic cardiovascular diseases. Quercetin has exhibited anticancer potential against a wide range of cancers such as prostate, lung, cervical, colon and breast by inhibiting cell explosion by causing cell cycle arrest ad apotosis **[4].**

HRP is a heme-containing enzyme that uses phenolic compounds and a hydrogen peroxide to create large reactive hydroxyl radicals **[5, 6]**. HRP is a heme protein with 308 amino acid residues. The N-terminal residue is blocked by pyrrolidene carboxyl residuals that appear to be covered inside the polypeptide chain. The peptides were sequenced with C-terminal and exclusive of a serine residue, indicating a labile Asn-Ser peptide bond **[7, 8]**. HRP contains 6 lysine residues in its glycoprotein structure, which produces a colored, luminescent, or fluorimetric derived of the labeled molecule when incubated with an appropriate substrate. HRP conjugated to a labeled molecule allows it to be detected and quantified **[9]**.

HRP exists in 30 isoforms due to presence of 18 % carbohydrates. The most predominant is isoenzyme C called (HRPC). This isoenzyme C is a monomeric glycoprotein as eight oligosaccharide side chains containing of 308 residues. HRP can bind to a substrate or suspended into the contaminant solution, get activated and then

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transform a variety of chemical pollutants as well as pharmaceuticals including chlorinated organic compounds, textile industrial dyes, effluents and oils **[10-12]**. The mechanism of Peroxidase catalyzes is heterolytic cleavage of the RO-OH bond which is based on the crystal structure of cytochrome-C peroxidase proposed by **[13]**. The versatility of this enzyme is additional underscored by its stability over a huge range of temperatures and pH **[14].** HRP is highest in many respects for these applications because it is minor, high stable, and lower expensive than other popular alternatives such as alkaline phosphatases.

Peroxidase isoenzymes play important role in plants. Peroxidase activity is measurable all the way through the whole lifespan of plants with a wide variety of reactions **[15]**. The recently identified HRP isoenzyme sequences **[16]** will facilitate future investigation on the expression patterns of individual isoenzymes in multiple plant tissues along with response to exterior stimuli. Peroxidase enzyme stability towards exterior factor (e.g. peroxide species, temperature) plays an essential role in the biocatalysis and bioremediation **[17]** activities. An acidic HRP isoenzyme A2 has been found more stable towards H_2O_2 inactivation than an isoenzyme C **[18]**. The isoenzyme E shows high specific activity in oxalacetate oxidation than the isoenzymes A2 and C **[19]**. In current days recombinant technology enables us to develop and combine such features within the HRP toolbox, allows development of novel and improved biocatalysts in the near future. The docking of the mutated protein model with known inhibitors or modulators helps in predicting the best possible stable 3D structure along with understanding critical molecular interactions of effluent molecules at the active site region.

Methodology:

Homology modeling

The amino acid sequence of Horseradish peroxidase was retrieved from NCBI. Multiple mutations were performed to already existing protein "Chain A, Recombinant horseradish peroxidase C Complex with ferulic Acid" on the basis of multiple sequence analysis and conserved regions. A sequence similarity search was performed individually in Protein Data Bank (PDB) using Protein BLAST tool for identifying templates for homology model building **[20]**. The sequence was searched for their structural similarity with the query mutant protein by running NCBI protein BLAST against Protein Data Bank (PDB) **[20]**. The template was identified on the basis of maximum score, smaller the e-value, >30% identity. 1PA2 protein was selected as a template for modeled protein with 56%identity. A comparative sequence alignment was performed with the template structures using ClustalX tool and online ClustalW tools. Pair wise sequence alignment of mutant protein and template obtained was shown in **Figure 1.**

```
gi|                        -QLTPTFYDTSCPNVSTIVRDTIVNELRSDPRIAASILRLHIHDCFVSGCDASILLDNTT
gi|11513747|pdb|1PA2|A     MQLNATFYSGTCPNASAIVRSTIQQALQSDTRIGASLIRLHFHDCFVNGCDASILLDDTG
                            **. ***. :***.*:***.** : *:** **.**::***:*****.*********:*

gi|                        SFRTEKDAFGNANSARGFPVIDRMKAAVESACPRTVSCADLLAIAAQTSVTLAGGPSWRV
gi|11513747|pdb|1PA2|A     SIQSEKNAGPNVNSARGFNVVDNIKTALENACPGVVSCSDVLALASEASVSLAGGPSWTV
                           *:::**:*  *.****** *:*.:*:*:*.*** .***:*:**:*:::**:******* *

gi|                        PLGRRDSLQAFPDLANATLPAPFFTLPQLKDSFRNVGLDRSSDLVALSGGHTFGKNQCRF
gi|11513747|pdb|1PA2|A     LLGRRDSLTANLAGANSSIPSPIESLSNITFKFSAVGLNT-NDLVALSGAHTFGRARCGV
                            ******* *    **:::*:*: :* ::. .*  ***:  .*******.****: :* .

gi|                        IMDRLYNFSNTGLPDPTLNTTYLQTLRGLCPLNGTLSALVDFDQRTPTIFDNKYYVNLEE
gi|11513747|pdb|1PA2|A     FNNRLFNFSGTGNPDPTLNSTLLSTLQQLCPQNGSASTITNLDLSTPDAFDNNYFANLQS
                           : :**:*** ** ******:* *.**: *** **: *::.::*  **  ***:*:.**:.

gi|                        QKGLIQSDQELFSSPNATDTIPLVRSFANSTQTFFNAFVEAMDRMGNITPLTGTQGQIRL
gi|11513747|pdb|1PA2|A     NDGLLQSDQELFSTTG-SSTIAIVTSFASNQTLFFQAFAQSMINMGNISPLTGSNGEIRL
                           :.**:********:   :.** :* ***..   **:**.::* .****:****::*:***

gi|                        NCRVVNSTS
gi|11513747|pdb|1PA2|A     DCKKVNGS-
                           :*: **.:
```

Figure 1: Conserved domain analysis of mutant HRP. The pair wise sequence alignment of the mutant HRP and the template 1PA2. It shows 56% of identity, 73% similarity. The template was selected for homology modeling considering the lowest E-value with the query sequence and >30% identity and the resolution of the protein (1.45 Å).

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MODELLER 9.14 was used along with an automated approach to comparative modeling by satisfaction of spatial restrains **[21]**. To develop models. After manually modifying the alignment input file in MODELLER 9.14 to match template and query sequence, 20 models were generated and all were thermodynamically minimized using molecular dynamics and simulation approach. By using MODELLER9.14 automodel class, calculated three dimensional models of the target automatically. The Lowest Objective Function is used toselect the best model by the smallest value of normalized Discrete Optimized Molecule Energy (DOPE) score. These models were then checked in detail for protein structure stereochemistry including Ramachandran plot and Psi/Phi angles using PROCHECK **[22]**.

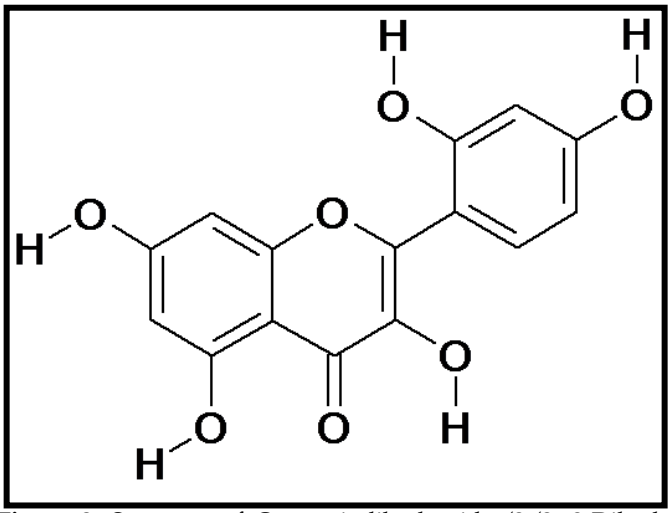

Figure 2: Structure of Quercetindihydroxide (2-(2, 2-Dihydroxy-phenyl)-3,5,7-trihydroxy-chromen-4-one)

Molecular docking studies

The structure of Quercetindihydroxide IUPAC Name: (2-(2,2-Dihydroxy-phenyl)-3,5,7-trihydroxy-chromen-4-one) shown in **(Figure 2)**. Horseradish peroxidase inhibitor (CID5280343) was retrieved from NCBI PubChem compound database. It was imported to SYBYL6.7 **[23]** and structure was energetically minimized by adding Gasteiger-Huckel charges. The molecule was then saved in .mol2 format for molecular docking purpose.

The 3D homology model was imported to Autodock 4.2 and structurally optimized by adding polar hydrogens to protein allocated with kollaman charges **[24]**. The model was saved in PDBQT format. Potential binding site for the model was identified using 3Dligand site. A grid was generated around to identify xyz coordinates (X-30.840, Y-44.520 and Z-8.139) around binding site of Horseradish peroxidase protein model. Optimizing the torsion angles and saving them in PDBQT format prepared ligands. Lamarckian genetic algorithm (LGA) was selected for freezing, docking and default parameters used in Autodock version 4.2.

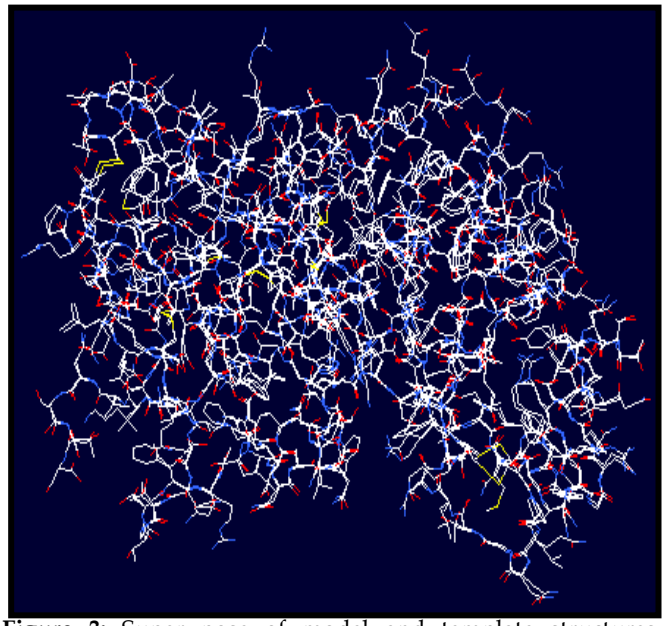

Figure 3: Super pose of model and template structures with backbone trace. The models were superimposed by using swisspdb viewer (spdbv). The model shows RMSD value of 0.38 Å.

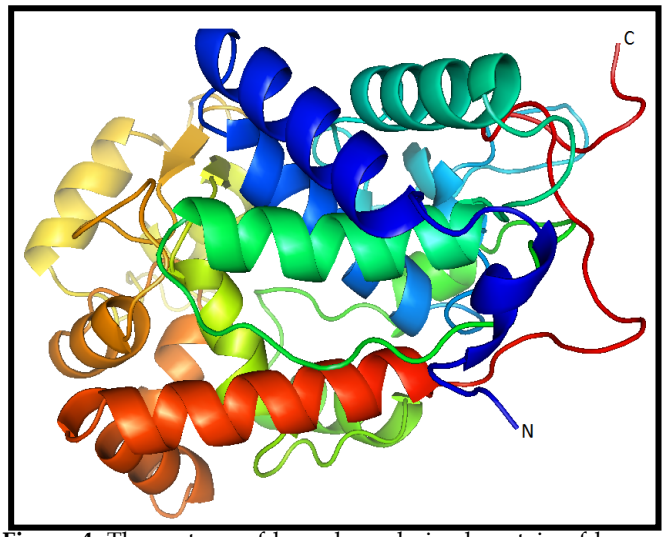

Figure 4: The cartoon of homology derived protein of horseradish peroxidase with N terminal and C terminal

Electrostatic distribution of the modeled surface

The electrostatic potential distribution of the modeled 3D structure of HRP was analyzed by UCSF Chimera **[25]**, a highly extensible

programme for analysis of molecular structure. It uses C++ code for color calculations. Electrostatic surface mapping of HRP was performed for a distribution and charge related properties of molecules and the surface of HRP was color coded as per the Coulomb's law.

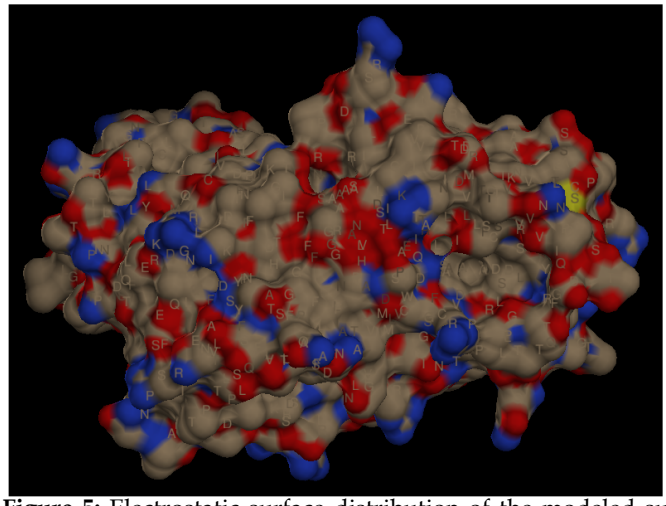

Figure 5: Electrostatic surface distribution of the modeled surface (by UCSF Chimera) the surface were color coded as per the standard protocol of UCSF Chimera, each amino acids were marked with standard code (blue for positive potential, white for neutral potential and red for negative potential).

ASA versus residue number plot

Accessible surface area of amino acid residues in a protein helps for localization of active sites. A characteristic 2D spiral plot of solvent accessibility provides a convenient graphical view of residues in terms of their exposed surface areas. In addition sequential plots of bar charts are also provided by the tool for each amino acid residues with the color coding corresponding to their location i.e. either in the surface or in the core. The ASA plot of HRP was prepared using ASA-View, a database and tools for the solvent accessibility representation in proteins **[26].**

Results and Discussion:

Horseradish peroxidase shows after sequence alignment and homology modeling highly conserved amino acids. The most homologous template for building a homology model for Horseradish peroxidase was identified through protein blast algorithm. Based upon the homology search, *Arabidopsis thalianaperoxidase* A2 (PDB entry: 1AP2) was selected as template. Twenty models were generated using Modeler 9.14 program. The alignment file was tweaked manually to excellent fit in the sequences. After the generated models for all the primary sequences, the model with the least object function was selected for further protein stereochemistry evaluation (phi and psi angles) with Procheck software. The PROCHECK software generates a number of files which list complete residue by residue data and the assessment of the generally excellence of the producing structure as compared to well refined structures of the same resolution **[27]**.

The Ramachandran plot of the 1AP2 shows 242 amino acid residues (90.0 %) in most favorable regions with 25 amino acid residues (9.3 %) falling into additionally allowed regions and with two amino acid residue (0.7 %) falling into the generously allowed regions**,** whereas for the modeled protein shows, 251 amino acid residues (92.6 %) in the most favorable region, 16 amino acid residues (5.9 %) in the additionally allowed region, 4 amino acid residues (1.5 %) in the generously allowed region. There is no amino acid residue present in disallowed region. These results clearly indicate that the generated model is more conformationally superior to the template structure. The modeled structure **(Figure 3)** was superimposed with the template 1PA2 by using SPDBV, it was observed that RMSD value on superposition of the modeled structure of HRP with the template structure was 0.38 Å. Cartoon form for the structure was given in (**Figure 4).**

Electrostatic surface distribution of the modeled surface (by UCSF Chimera) the surface were color coded as per the standard protocol of UCSF Chimera, each amino acids were marked with standard code (red for negative potential, to white near neutral, to blue for positive potential). UCSF Chimera showed that HRP protein has more positive charge residues displayed in blue color **(Figure 5),** on the outer surface suggesting the fact that the residues are the part of the conserved domain which have specific functionalities i.e. receptor binding **[28]**.

ASA describes structure stability receptor binding mode of the protein. ASA vs residue number plot by ASA-View the colors are coded as Blue for Positive charged residues (R, K, H), Redfor Negative charged residue (D, E), Green for Polar uncharged residues (G, N, Y, Q, S, T, W), Yellow for Cystein and Gray for Hydrophobic residues (All others) for both model and template. Relative solvent accessibility plots in the original order of the residues of both model and template was illustrated in **(Figure 6).**

AutoDock 4.2 was used for molecular docking studies. During the docking procedure, the program selects only best fit active site pocket with respect to the ligands in order to dock them. Results obtained from AutoDock 4.2 **[29]** provided information on the binding orientation of ligands at the active site region. The docking programs place both the protein and ligand molecule in various orientations; conformational positions and the lowest energy confirmations, which are energetically favorable, are evaluated and analyzed for interactions **[30]**. Free energies of binding (ΔGb) and dissociation constants (Ki) as calculated by AutoDock are summarized.

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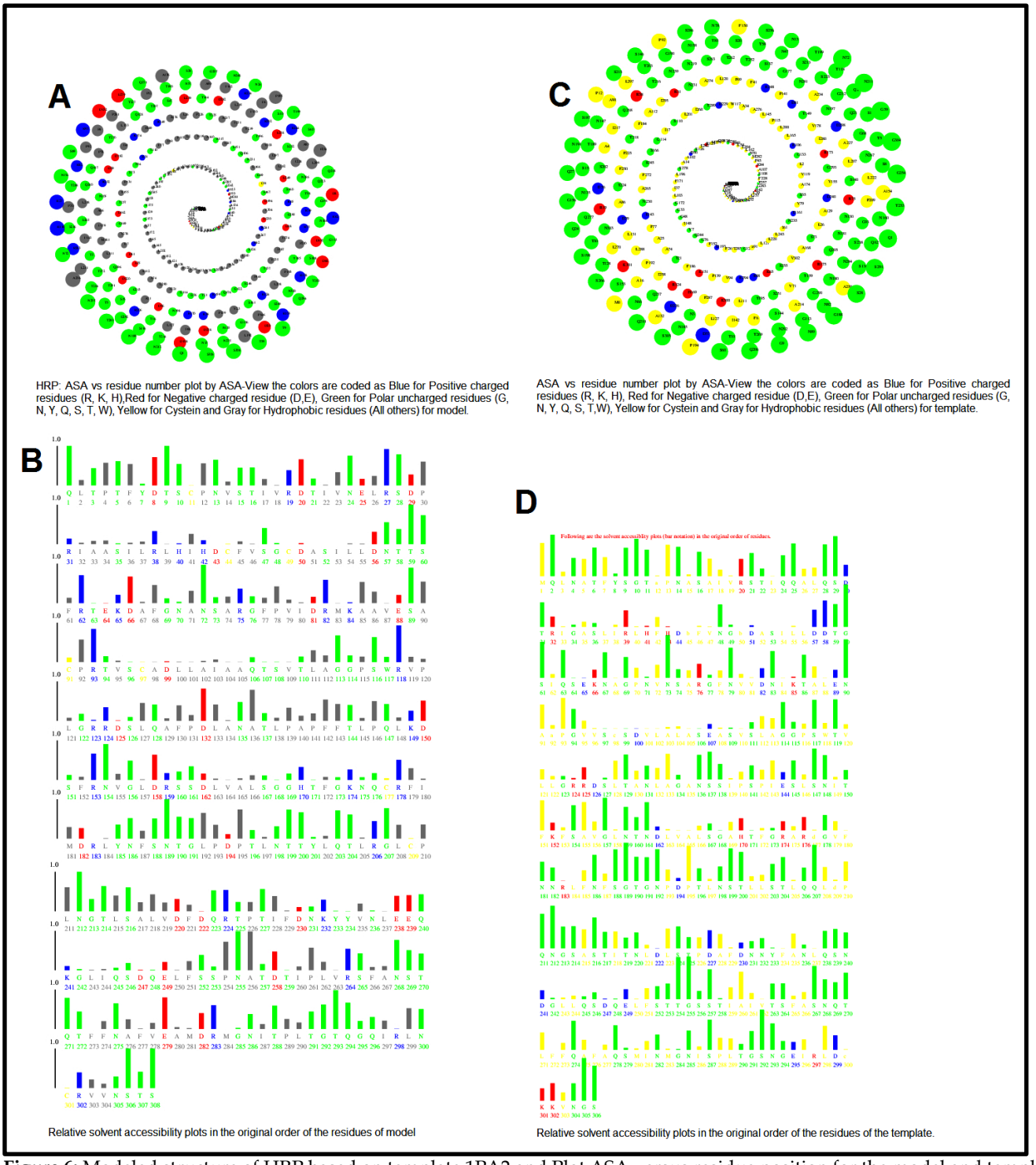

Figure 6: Modeled structure of HRP based on template 1PA2 and Plot ASA versus residue position for the model and template

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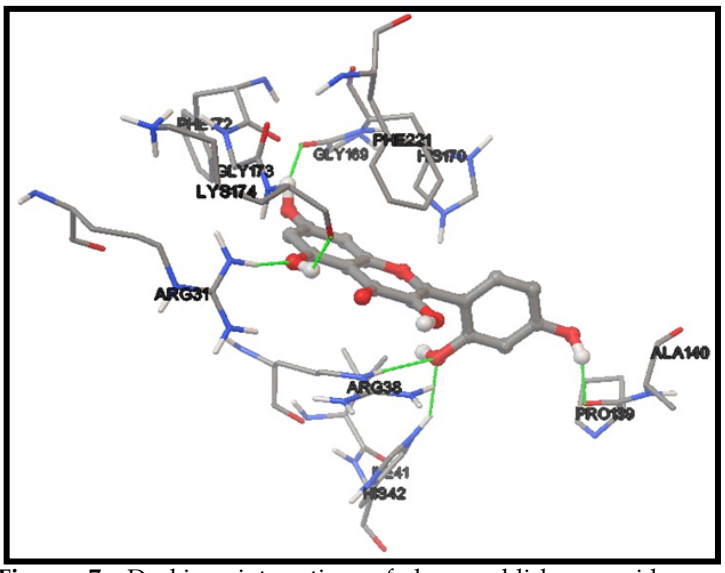

Figure 7: Docking interaction of horseraddish peroxidase with Quercetindihydroxide

Quercetindi-hydroxide was docked with the HRP homology model The docking interaction indicates six critical molecular interactions as shown in **(Figure 7)**. Docking study with Quercetindihydroxide has shown hydrogen bonding with P139, H42, A31, L174, A38, and G169 residues with a docking score of -7.67 kcal/mol.

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