

Molecular docking insights into the inhibition of laccase activity by medicarpin

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Abstract Molecular docking calculations were performed to understand the inhibition mechanism of medicarpin on the lignin degradation enzyme laccase. The study was carried out in two stages. The first stage was performed on a laccase–oligolignol complex, to elucidate the active binding mode in the enzyme that initiates lignin degradation. The second stage was performed on a laccase–medicarpin complex, to understand the inhibitory effect of medicarpin on the enzymatic mechanism. In this two-step procedure, the crystal structure of a laccase (PDB: 1GYC) obtained from *Trametes versicolor*, a white-rot fungus capable of degrading lignin in wood, was employed because it produces considerable amounts of laccase. The results obtained from the laccase–oligolignol complex indicate that the binding site is located in a pocket that surrounds the T1 copper atom, which is in agreement with experimental reports. The results from the laccase–medicarpin complex indicate that the inhibitory effect occurs by the blockage of a solvent channel in the *T. versicolor* laccase by a medicarpin conformer, preventing the rapid access of molecular oxygen to the trinuclear T2/T3 copper cluster, and also by the coupling of medicarpin conformers in the T1 site pocket, blocking access by oligolignol molecules. Therefore, this coupling of medicarpin to two sites in laccase interrupts the enzymatic mechanism of lignin degradation. The results of this study may be useful in developing wood-preserving formulations based on medicarpin or similar molecular structures and in expanding the scientific knowledge of laccase activity for biotechnological applications.

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Introduction

The most characterized laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are from the tree *Rhus verniciflua* (also called the lacquer tree, hence the name laccase) (Yoshida 1883) and from the fungus *Trametes versicolor* (Bertrand et al. 2002). This basidiomycete plays a major role in the degradation of lignin and has the capacity to oxidize recalcitrant aromatic compounds with high redox potentials (Thurston 1994). This capacity has been the subject of interest in biotechnological applications such as pulp delignification and bioremediation of soils and water (Arora and Sharma 2010), bearing in mind that laccase is a monomeric copper enzyme capable of oxidizing substrates that vary from ascorbic acid, amines, and phenolates to phenolic lignin degradation intermediates. Laccase catalyzes the four-electron reduction in molecular oxygen to water using one-electron donors (Nakamura and Go 2005) and belongs to the family of blue copper oxidases, which include ascorbic oxidase, ceruloplasmin, and other less well-characterized enzymes (Thurston 1994).

The laccase from the ligninolytic fungus *T. versicolor* contains four copper atoms per functional unit, which are classified as type 1 (T1, with one copper atom), type 2 (T2, with one copper atom), and type 3 (T3, two copper atoms), according to their spectroscopic and redox properties (Wong 2009; Xu et al. 1996). The enzyme catalyzes the overall reaction $4RH + O_2 \rightarrow 4R + 2H_2O$; hence, the stoichiometry comprises four molecules of reducing substrate for every oxygen molecule, and in total, four electrons are transferred to O_2 . The first step is reduction in the substrate by copper (Cu^{2+} to Cu^+) at the T1 site, an electron acceptor. The electrons from the substrate are transported to the trinuclear T2/T3 sites, where the conversion of the fully oxidized form of the enzyme to its reduced form takes place (Wong 2009; Solomon et al. 2001).

The molecular structure of fully active laccase is known (Briozzo et al. 2002), resulting in the characterization of the sites of the four copper atoms. The T1 copper lies embedded in a wide hydrophobic binding pocket, about 6.5 Å below the surface of the enzyme, rich in π electron density, where a range of organic substrates can bind and undergo rapid one-electron oxidation to radical products that separate from the enzyme before further reaction. The T2 and T3 copper atoms form a trinuclear cluster (T2/T3) at the interface of sites 1 and 3, where an oxygen molecule is reduced by electrons transferred from the T1 copper atom (Wong 2009) via a combination of the peptide chain and hydrogen bonds. Solomon et al. (2008) reported that the closest distance between the T1 and T2/T3 copper atoms is around 13.0 Å. The site for oxygen reduction at the T2/T3 cluster is accessible to solvent from two channels, which lead to the T3 coppers and the T2 copper, respectively. The T2 copper is more exposed compared with the two T3 copper atoms. The three Cu atoms of the T2/T3 cluster are close to each other, in an almost equilateral triangle with an average Cu–Cu distance of 3.85 Å. It appears that the two solvent channels of the *T. versicolor* laccase are located so as to allow rapid access of molecular oxygen to the trinuclear copper cluster and subsequently release of

product water (Solomon et al. 2008). Figure 1 shows the interaction sites in the enzyme studied in this work.

There are several current biotechnological applications of laccase (Giardina et al. 2010); therefore, it is essential to know how different compounds affect its enzymatic action, with special interest for in situ treatment technologies. In particular, *T. versicolor* has been considered a very aggressive white-rot fungus, which is able to degrade lignin in wood, because it produces considerable amounts of laccase (Tanaka et al. 1999). White-rot fungi also participate in the degradation and decomposition of xenobiotic pollutants (Suresh et al. 2008). Knowledge of the response of laccase to the presence of organic compounds is necessary to determine its effectiveness in soil and water bioremediation technologies.

The authors have previously reported on the antifungal activity of heartwood extracts from *Dalbergia congestiflora* Pittier against *T. versicolor* (Martínez-Sotres et al. 2012), the standard basidiomycete fungus for wood decay laboratory studies (ASTM 1998). The antifungal component was isolated and identified from hexane extract as medicarpin, which is a phytoalexin in many legume species and is the most widespread pterocarpan (Kuc 1995). The hexane extract was added to culture media to evaluate the effect on the laccase activity of *T. versicolor*; laccase activity was not observed in the presence of medicarpin (Martínez-Sotres 2011). Based on these results, it is necessary to study the interactions between laccase and relevant compounds (lignin and medicarpin) at the molecular level. Molecular understanding of the inhibition of laccase activity by medicarpin is necessary for developing wood-

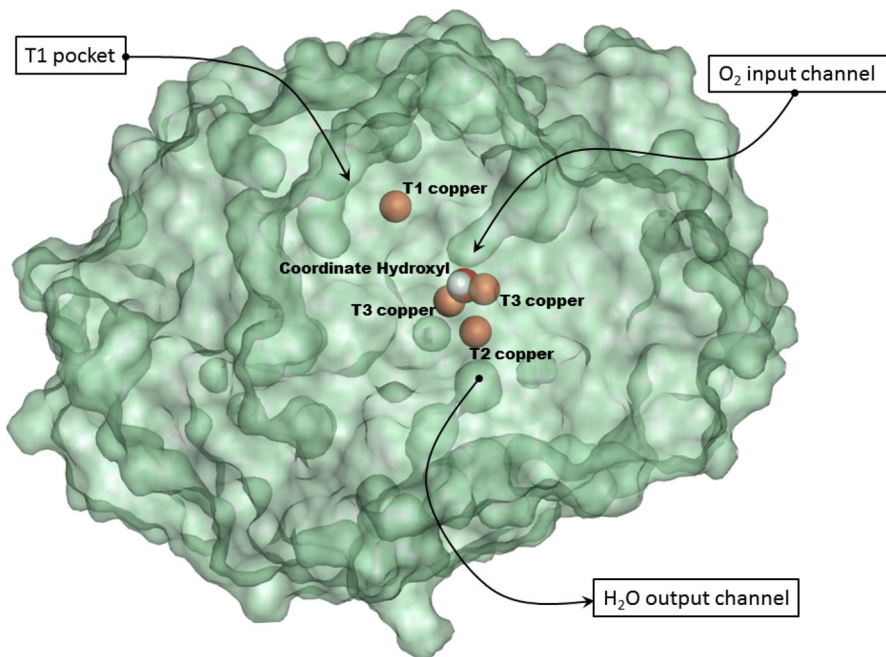


Fig. 1 Molecular surface based on the water access radii of laccase. Overview of the crystal structure of *Trametes versicolor* laccase (PDB entry 1GYC) (Bertrand et al. 2002)

preserving formulations based on medicarpin or similar molecular compounds, and in elucidating laccase activity for biotechnological applications.

The objective of this study is to describe at the molecular level how medicarpin inhibits laccase activity. First, a computational molecular docking methodology was used to analyze the initial step of the catalytic mechanism of *T. versicolor* laccase, by assessing the binding of spatially and energetically coupled conformations of lignin models (dimers and trimers). To the authors' knowledge, there are no systematic studies at the molecular level describing how laccase couples to lignin or oligolignols for their degradation. After locating the laccase–lignin complex that initiates the catalytic oxidation of the lignin models, the inhibitory effect of medicarpin was elucidated when it couples to laccase, using the same computational molecular docking methodology. These results provide insights into the inhibitory effect of medicarpin on *T. versicolor* laccase.

Materials and methods

The X-ray structure of the *T. versicolor* laccase enzyme co-crystallized with isopropyl alcohol (PDB ID: 1GYC) (Piontek et al. 2002) was taken from the Brookhaven Protein Data Bank (Berman et al. 2000). Ligands were removed, as were water molecules except for a hydroxyl coordinated to the two copper atoms at the T3 site. The hydrogen atoms in the protein were added using the PSFGEN program included in the VMD 1.8.6 package (Humphrey et al. 1996).

This study was carried out in two parts: in the first part, the laccase–oligolignol complexes were generated. Lignin was modeled using two of its oligolignol substructures, a dilignol and a trilignol (Fig. 2). The dilignol lignin substructure was built by bonding two CA units with a β -O-4' linkage, and the trilignol lignin substructure was built by combining three CA units via β -O-4' and β '-5'' linkages.

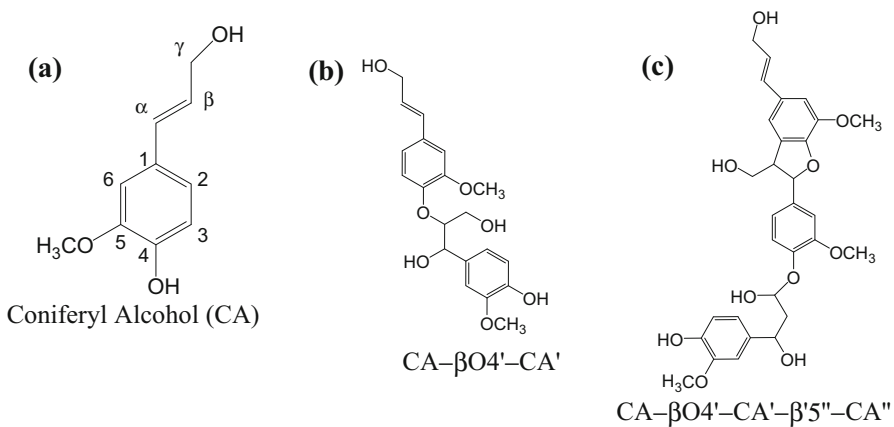
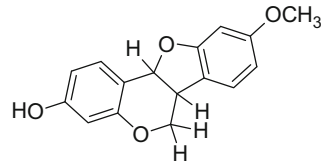


Fig. 2 Models of lignin substructures considered as substrates of the 1GYC laccase in the docking study. **a** The polymeric unit: coniferyl alcohol (CA) numbered according to Freudenberg (1965); **b** the dilignol model; and **c** the trilignol model

Fig. 3 Structure of (+)-3-hydroxy-9-methoxypterocarpan, also called medicarpin



These linkages were selected because they are the most frequent type of bonds in softwood and hardwood lignins (Glasser and Sarkanen 1989). The medicarpin molecular structure (Deesamer et al. 2009) is shown in Fig. 3. All chemical structures for the laccase substrates (ligands), and their three-dimensional structures, were built using the ChemBioOffice ultra software, version 11.0.1 (CambridgeSoft 2011). Medicarpin was built from geometric data reported by Deesamer et al. (2009); meanwhile, the lignin substructures were built considering the geometric data of Durbeej and Eriksson (2003).

The molecular docking procedure for obtaining the binding energy between the laccase enzyme model and its substrates was carried out for each ligand in the three-dimensional structure of the laccase enzyme, 1GYC. The initial search for binding sites was performed using AutoDock 4.2 software (Morris et al. 2009), developed to provide a computational scheme for obtaining the binding energies of ligands with large molecular targets in a docking system. The initial configurations in the docking simulations were built by considering the ligands far away from the 1GYC macromolecule, and their flexibility was limited to six torsional degrees of freedom, selecting torsions that allowed the fewest number of atoms to move (freezing the core of the molecule). AutoDock includes several methods for carrying out a conformational search; however, the Lamarckian genetic algorithm (Morris et al. 1998) provides an effective search for broad applications and was selected for use in this study. The Lamarckian genetic algorithm was used for all the molecular docking calculations with the following parameters: a random population of substrate conformations in up to 250 arbitrary orientations, a mutation rate of 0.02, and a crossover rate of 0.8. Simulations were carried out considering 2.5 million energy evaluations with a maximum of 25,000 generations. Each simulation was performed 600 times, generating 600 docked conformations. The ligand preparation process included in AutoDock automatically assigned the number of permitted torsions for the lignin substructures and medicarpin, allowing flexible structures in the molecular docking calculation. The space where dilignol, trilignol, and medicarpin moved over the 1GYC laccase model was fixed, considering the enzyme centered inside a grid box comprised of $126 \times 126 \times 126$ grid points with a spacing between each grid point of 0.385 Å. Figure 4 shows the grid box for the 1GYC laccase structure used in the conformational search.

The binding modes search was carried out by taking advantage of AutoDock in allowing flexible residues in the enzyme. The flexible amino acids were selected considering the crystallographic data reported by Lyashenko et al. (2006) on a laccase structure with the same amino acids as *T. versicolor* laccase in the T1 and T2/T3 interaction sites. Flexible docking was performed allowing three torsions in residues ALA80, GLN102, SER110, HIS111, SER113, TYR116, PHE162,

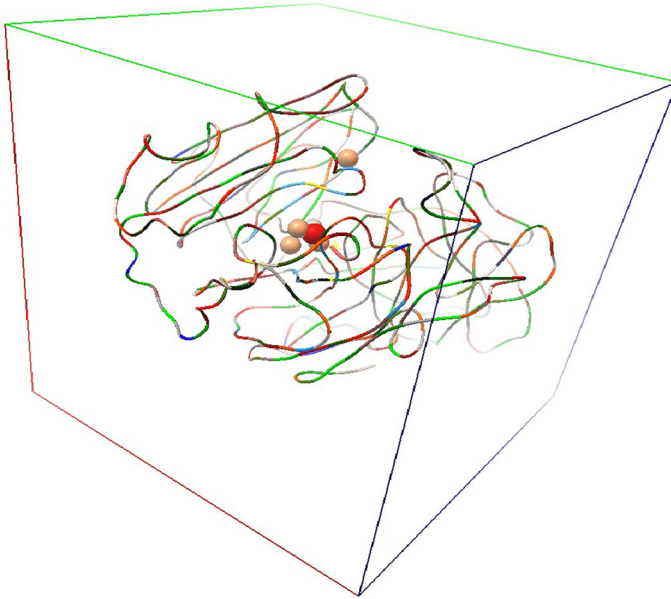


Fig. 4 Grid box surrounding the laccase three-dimensional enzyme model, where the oligopeptides are placed into the docking simulation. The amino acids sequence is displayed using the tube style included in AutoDockTools (Sanner et al. 2007)

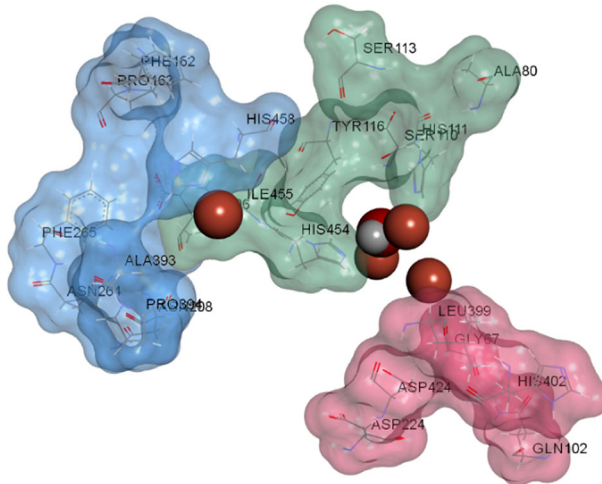


Fig. 5 Three-dimensional structure showing the flexible laccase residues at the active sites. Interaction sites are displayed considering the van der Waals radii surfaces for the corresponding oligopeptides. The T1 pocket is on the *left*, the O₂ access channel is in the *middle*, and the H₂O output channel is on the *right*

PRO163, ASP206, ASN208, ASP224, ASN264, PHE265, ALA393, LEU399, ASP424, HIS454, ILE455, and HIS458. Figure 5 shows the flexible protein residues selected for this molecular docking study.

By default, the implemented force field in AutoDock does not include parameters for copper atoms or their ionic forms. Thus, the file AD4_parameters.dat was modified to include copper parameters according to the instructions in the AutoDock 4.2 user guide.

The second part of this study focused on elucidating the inhibitory effect of medicarpin through its binding energy and binding conformations, using the same computational molecular docking methodology, to compare them with the molecular docking results obtained with the dilignol and trilignol substrates.

Results and discussion

Binding modes for the lignin models inside the laccase of *Trametes versicolor*

It is expected that molecular docking calculations between dilignol and trilignol with the 1GYC laccase structure should generate complexes consistent with reports from the literature that the first step in the catalytic mechanism of laccase is the oxidation of lignin components by the copper ion at the T1 site. Figure 6 displays the 600 calculated conformers of dilignol coupled to the 1GYC laccase overlaid on the enzyme's structure. A large number of dilignol conformers show an affinity for the cavity where the T1 copper is located (Table 1); however, this is not the only coupling site. In the course of docking calculations, coupling also occurred around the 1GYC molecular structure. Dilignol coupled conformations away from the T1 copper site, would not promote laccase activity.

The best docking result (the lowest binding energy) obtained for dilignol occurs inside the hydrophobic binding pocket where the T1 copper is lying embedded

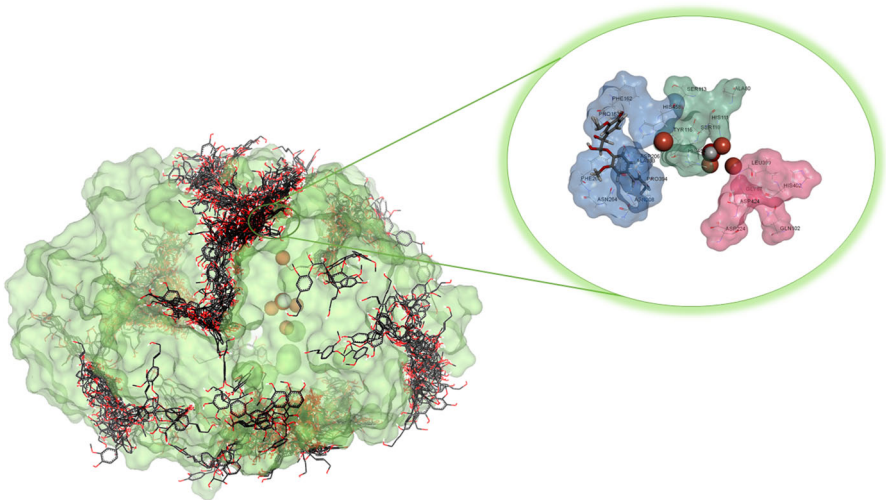
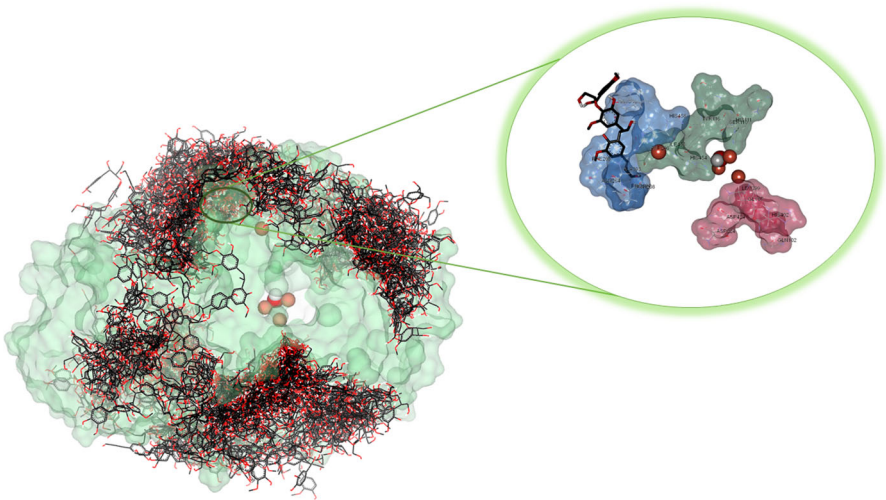


Fig. 6 Docked conformations of dilignol over the laccase 1GYC model, highlighting the strongest binding energy complex. The conformers only include polar hydrogens

Table 1 Binding energies obtained from AutoDock calculations, summarizing the corresponding binding modes for dilignol and trilignol at known interaction sites

Substrate	Number of docked conformers and their localization	Cluster mean binding energy (kcal/mol)	Lowest binding energy (kcal/mol)
Dilignol	197 at T1 site	-6.39	-7.22 at T1 site
CA- β O4'-CA'	22 inside O ₂ channel	-5.33	
	148 inside H ₂ O channel	-5.40	
Trilignol	128 inside T1 site	-1.29	-6.00 at T1 site
CA- β O4'-CA'- β '5''-CA''	46 inside O ₂ channel	-1.04	
	163 inside H ₂ O channel	-1.12	

**Fig. 7** Docked conformations of trilignol over the laccase 1GYC model, highlighting the strongest binding energy complex. The conformers only include polar hydrogens

(Table 1), and where the largest group of docked conformations occurred. This cluster of dilignol conformers has a stronger binding energy than those bound at other sites over the laccase.

Figure 7 displays the 600 docked conformations obtained for the trilignol, which show similar behavior to the results obtained for dilignol (Table 1). However, the cluster with the largest number of trilignol conformers does not include a conformation with the strongest binding energy for trilignol.

Table 1 shows the AutoDock binding energy values obtained in the docking simulations, corresponding to the binding modes for dilignol and trilignol at different sites. Dilignol achieves a lower binding energy than trilignol when it couples with the 1GYC laccase.

Even though molecular docking results show that several conformers of dilignol and trilignol couple inside the molecular oxygen and water channels, their unfavorable binding energies relative to binding to the T1 site hinder the formation

of a stable complex in the docking process. The ability of 1GYC laccase to degrade lignin components can be explained using a thermodynamic framework, because the molecular docking simulations show that the 1GYC laccase can couple to and stabilize the lignin components at the T1 site. The binding energy results for the triligol model could be explained in terms of steric effects, which promote repulsive interactions. Therefore, laccase could be selective in the catalysis of lignin fragments where lignin folding is absent.

The laccase–oligolignol complexes generated in the first part of the study are consistent with binding modes reported from experimental data, yielding insights into the initial steps of lignin degradation by *T. versicolor* laccase. Therefore, the binding mode of dilignol and triligol molecules to, and their molecular interactions with, the 1GYC laccase may be considered a reference for understanding the laccase–oligolignol coupling that initiates the catalytic mechanism.

Inhibitory binding modes of medicarpin

The molecular docking simulations of medicarpin with the 1GYC laccase revealed very interesting results. Three tightly grouped clusters were obtained: a cluster composed of 161 coupled conformers that occurred at the T1 site, a second cluster containing 82 conformers that occurred inside molecular oxygen channel, which included the strongest binding energy conformer, and a set of 225 conformations clustered in the H₂O exit channel (Table 2; Fig. 8).

The medicarpin docked conformers indicated the presence of three binding modes that can produce inhibitory effects in 1GYC laccase: (1) obstructing the T1 site to restrict the binding of lignin components, (2) interfering with the access of molecular oxygen to the T2 and T3 sites, preventing its reduction to water, and (3) obstructing the release of product water from the active site, since this was the largest group of complexes (225) and the binding energy was stronger than for medicarpin binding to the T1 site.

From these results, it appears that the inhibitory effect of medicarpin on the 1GYC laccase could be elucidated in terms of the distribution and spatial binding modes of conformers, in place of a thermodynamic framework. Medicarpin has a rigid structure with one torsional bond, minimizing its scattering over the laccase three-dimensional model, resulting in tight clusters of conformers.

Table 2 Binding energies obtained from AutoDock calculations, summarizing the corresponding binding modes for medicarpin at known interaction sites

Substrate	Number of docked conformers and their localization	Cluster mean binding energy (kcal/mol)	Lowest binding energy (kcal/mol)
Medicarpin	161 inside T1 site	−6.10	−7.35 inside O ₂ channel
	82 inside O ₂ channel	−7.24	
	225 inside H ₂ O channel	−6.38	

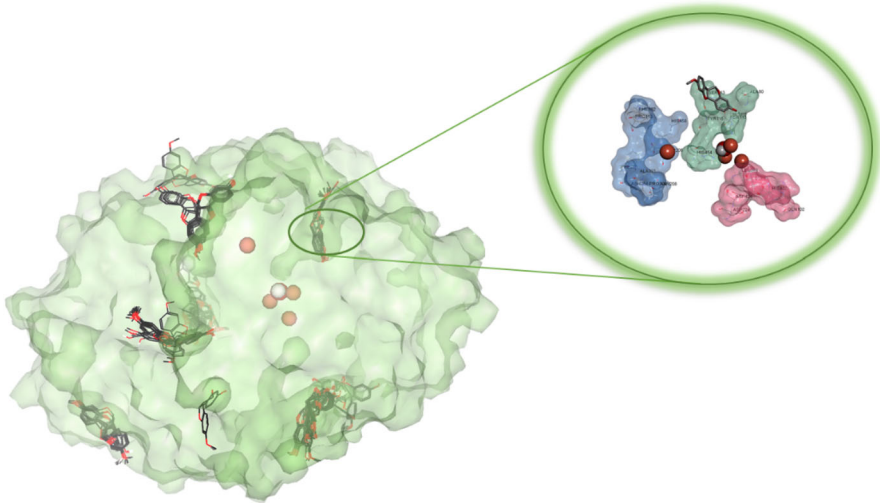


Fig. 8 Docked conformations of medicarpin over the laccase 1GYC model, highlighting the strongest binding energy complex. The conformers only include polar hydrogens

Conclusion

Spatial and energetic binding modes of lignin components in the 1GYC laccase to describe the initial step of the catalytic mechanism at the molecular level were obtained from molecular docking simulations. The results of this study indicate that the degradation of lignin by *T. versicolor* laccase begins in the hydrophobic binding pocket where the T1 copper lies embedded; lignin binding to this site is a thermodynamically favorable process. In the absence of a co-crystallized structure between lignin (or lignin components) and *T. versicolor* laccase, these results provide a molecular basis for understanding the initial steps in laccase activity. The binding modes of medicarpin to laccase provide insights into its inhibitory effect. The medicarpin structure enables favorable binding modes with the laccase enzyme that, in terms of the distribution of docked conformations, could hinder the lignin component binding at the T1 site and perturb the O₂ channel. The results obtained in this work could help scientists in designing new laccase enzyme inhibitors and to further investigate the enzymatic activity of laccase, based on interactions at the molecular level.

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