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Article

Laccase mediator systems: Virtual screening for Natural mediators

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Abstract: Industries are increasingly turning to green chemistry, with laccase enzymes garnering attention due to their ability to use oxygen to produce water as a by-product while catalyzing a wide range of reactions. Bacterial laccases, though less explored, offer stability at high temperatures and pH levels. However, they require mediator systems to handle complex molecules. Structure-based virtual screening aids in identifying suitable mediators, potentially reducing costs, and accelerating discovery. These techniques rely solely on the target protein three-dimensional structure and do not require knowledge of the unique bioactivity of the protein. As a result, structure-based approaches are theoretically more adaptive to unknown protein and ligand compounds than ligand-based approaches. Therefore, we aimed to screen and compare the efficacy of natural mediators over the artificial mediators for laccase oxidation. Here, we predicted that natural mediators like coumaric acid and ferulic acid showed promise comparable to artificial ones, such as N-hydroxythalimide and N-hydroxyacetanilide respectively, in computational assessments (binding energy), suggesting virtual screening's potential in mediator discovery.

Keywords: Laccase, Natural mediators, Artificial mediators, Affinity score

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1. Introduction

Laccases represent a group of multi-copper oxidases responsible for facilitating the oxidation of various substrates, notably phenolic derivatives [1]. These enzymes catalyze reactions wherein the substrate undergoes mono-electronic oxidation, resulting in the formation of the corresponding radical species. Simultaneously, molecular oxygen is reduced to water during these catalytic processes [2]. In numerous circumstances, complicated polyphenolic structures, non-phenolic chemicals, and aromatic amines, i.e. molecules with a high redox potential (>1.5 V) which present difficulties for laccases when it comes to direct catalysis of the oxidation reaction [3]. Furthermore, the active core of the enzyme is inaccessible to complex molecules, especially those found in lignocellulose complexes, indicating a poor affinity of the enzyme for the substrates. To address these issues, a mediator, typically a low molecular weight compound, can be employed [4]. Laccase-mediator systems have the capability to oxidize a diverse array of substrates, and this ability has been utilized in various applications, including the degradation of dyes [5], transformation of halogenated pesticides [6], oxidation of polycyclic aromatic hydrocarbons [7], and the degradation of endocrine-disrupting chemicals [8] and lignin [9]. Various synthetic compounds, including ABTS, TEMP, VIOL, and HBT, have been employed as laccase mediators in these biocatalytic processes. Nevertheless, the utility of these compounds is significantly restricted due to their high cost, the toxicity of certain by-products, and the need for a large mediator/substrate molar ratio [10]. Indeed, many of the tested

mediators have proven to be ineffective as they undergo complete oxidation and are exhausted from the cycle [11]. Consequently, these synthetic mediators have been used in molar ratios as high as 40 to 1 [12]. There is a suggestion that natural mediators could offer a more efficient, cost-effective, and environmentally friendly alternative.

The current exploration of mediators faces two fundamental challenges. Firstly, understanding how a molecule engages with its target, typically a protein like laccase, is essential [13]. Secondly, it's crucial to determine how the same molecule can effectively interact with laccase, considering that the enzyme's site of action is involved. Despite the extensive use of computational chemistry in biological and medical studies, there is a notable scarcity of such investigations in laccase-mediator systems. This scarcity can be attributed to limitations associated with computational chemistry studies in this context, primarily linked to the heterogeneity of available experimental data. Existing literature reveals variations in reaction conditions, including the mediator/substrate molar ratio, nature of the substrate, and the reaction time. So, this study used computational chemistry to explore laccase mediator systems, aiming to precisely identify binding locations and analyze how these sites can be efficiently targeted in virtual screening. This approach is crucial in the initial stages of discovering target ligands, enhancing the starting library with active substance. Virtual screening enables the rapid evaluation of thousands of compounds within hours, minimizing the need for synthesis or purchase and subsequent testing, thus cutting down on costs [14].

2. Materials and Methods

2.1. Data sources

A CotA laccase amino acid sequence of the strain *Bacillus sp.* NP157 was obtained from the National Center for Biotechnology Information (NCBI) genome database (https:www.ncbi.nlm.nih.gov). The list of natural and chemical mediators was downloaded from PubChem compound database [15].

2.2. Virtual screening of mediators' molecules

The 3D structure prediction of laccase was based on a homology modelling method using UCSF ChimeraX [16]. Furthermore, using AutoDock Tool version 4.1, the ligands and receptor protein were prepared. The processed receptor and ligand structures were docked blindly. Based on the least energy complex (enzyme and ligand) formation, the best docked complex was selected and was visualized with Biovia Discovery Studio 2021 client 21.1 (BIOVIA, San Diego, CA, USA).

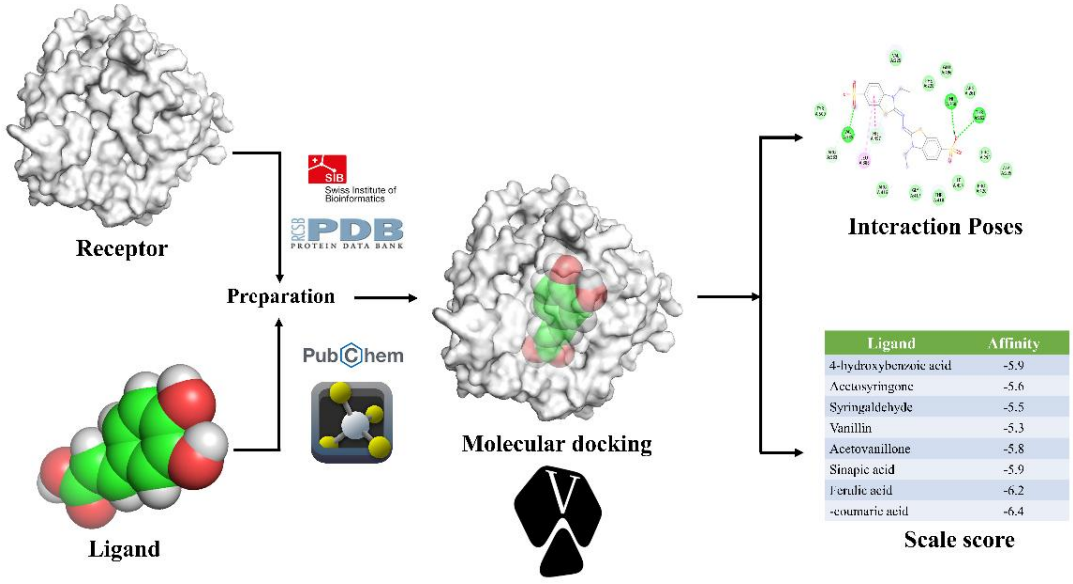

Figure 1. The workflow process of virtual screening for mediators.

3. Results

3.1. Enzyme structure prediction and docking with mediator molecules

In this software, AlphaFold's structure prediction generates initial protein models, albiet less accurately than expreimental ones. The 3D strucuure of CotA laccase was prediced based on the hightest sequence coverage and the local distance difference test (pLDDT) socre, which evaluates confidenece in he local structure and indicates regions of reliability within the predicted structure. Moleular docking experiments were conducted using AutoDock Tools and Vinna software to assess the interaction of 3D modeled laccase with both artifiical (2,2′-azino-bis(3-ethylbenzothaiazoline-6-sulfonic acid); 1-hydroybenzotriazole; Violuric acid; N-hydroxyacetanilide; 2,2,6,6-Tetramethylpiperidine-1-yl; N-hydroxyacetanilide) and natural (4-hydoxybenzoic acid; Acetosyringone; Syringaldehyde; Vanillin; Acetovanillone; Sinapic acid; Ferulic aid; Coumaric acid) mediators. Despite the widespread use of artificial mediators, we amed to explore environmentally friendly alternatives provided by natural mediators. Results obtained from this study were reported in Table 1, revealed higher binding efficiencies with artifiial mediators (-6.3 to -7.4) compared to natural ones (-5.3 to -6.4). Among the 14 mediators tested (6 artificial and 8 natural), artificial ones exhibited superior binding efficiencies, ranging from -7.4 with ABTS to -6.3 with HANAN. Conversely, natural mediators showed a maximum binding efficiency of -6.4 with coumaric acid and a minimum of -5.3 with both vanillin and acetovanillone.

Table 1. Theoretical Binding affinities of mediators

Artifiical mediators	**Binding energy (kcal/mol)**
ABTS (2,2′-azino-bis(3-ethylbenzothaiazoline-6-sulfonic acid)	-7.4
HOBt (1-hydroybenzotriazole)	-6.3
NHPI (N-hydroxypthalimide)	-6.5
VA (Violuric acid)	-6.8
NHAN (N-hydroxyacetanilide)	-6.3
TEMPO (2,2,6,6-Tetramethylpiperidine-1-yl)	-7
Natural mediators	**Binding energy (kcal/mol)**
HBA (4-hydoxybenzoic acid)	-5.9
AS (Acetosyringone)	-5.6
SA (Syringaldehyde)	-5.5
V (Vanillin)	-5.3
AV (Acetovanillone)	-5.3
S (Sinapic acid)	-5.8
FA (Ferulic acid)	-6.2
CA (Coumaric acid)	-6.4

3.2. Comparison of Natural and Artificial mediators based on binding affinity values

In comparison between artificical and natural mediators that binds to the predicted laccase, only two of the natural mediaotr i.e. Caumaric acid and ferulic acid concides with almost same binding score with N-hydroxybenzotriazole (-6.5 kcal/mol) and N-hydroxyacetanilide (-6.3 kcal/mol) (Fig. 2). While others are not observed in comparison level value of binding energy. Similarly the best dock complexed were observed for interaction posed with the predicted structure (Fig. 3). The best artificial mediator dock complexed showed the conventional hydrogen bond interaction with valine and threonine residues. Whereas in case of natural mediator, it showed conventional hydrogen bond interaction with two asparagine residues. Though the natural mediators

used in this study do not show the high binding efficiences compared to artificial mediators, coumaric acid and ferulic acid could be use in place of N-hydroxybenzotriazole and N-hydroxyacetanilide respectively.

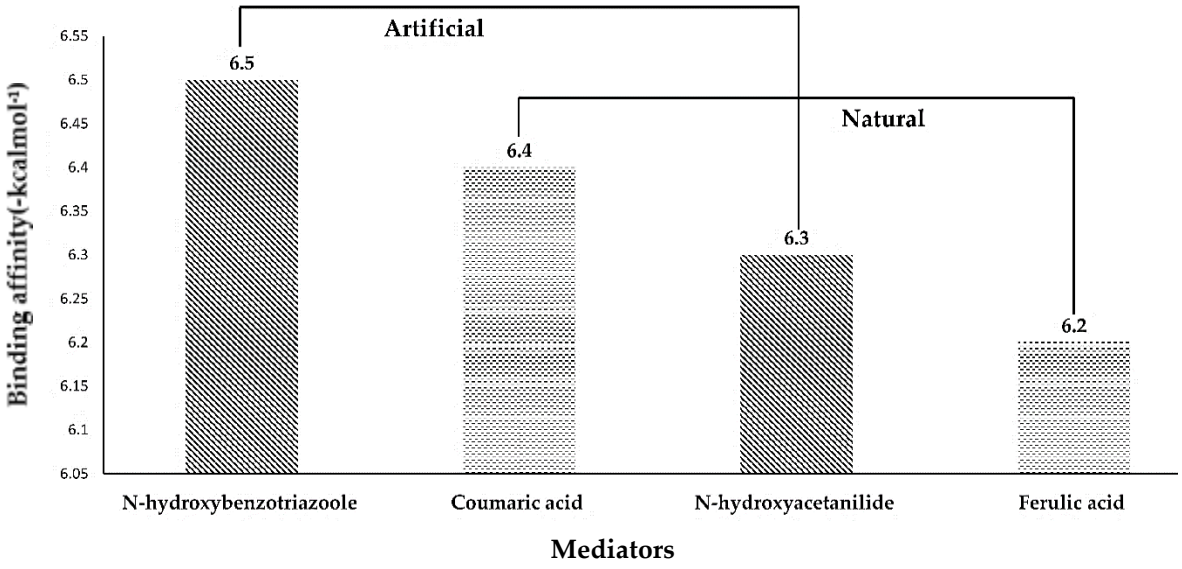

Figure 2. Binding affinity values (kcal/mol) of most comparable artificial and natural mediator.

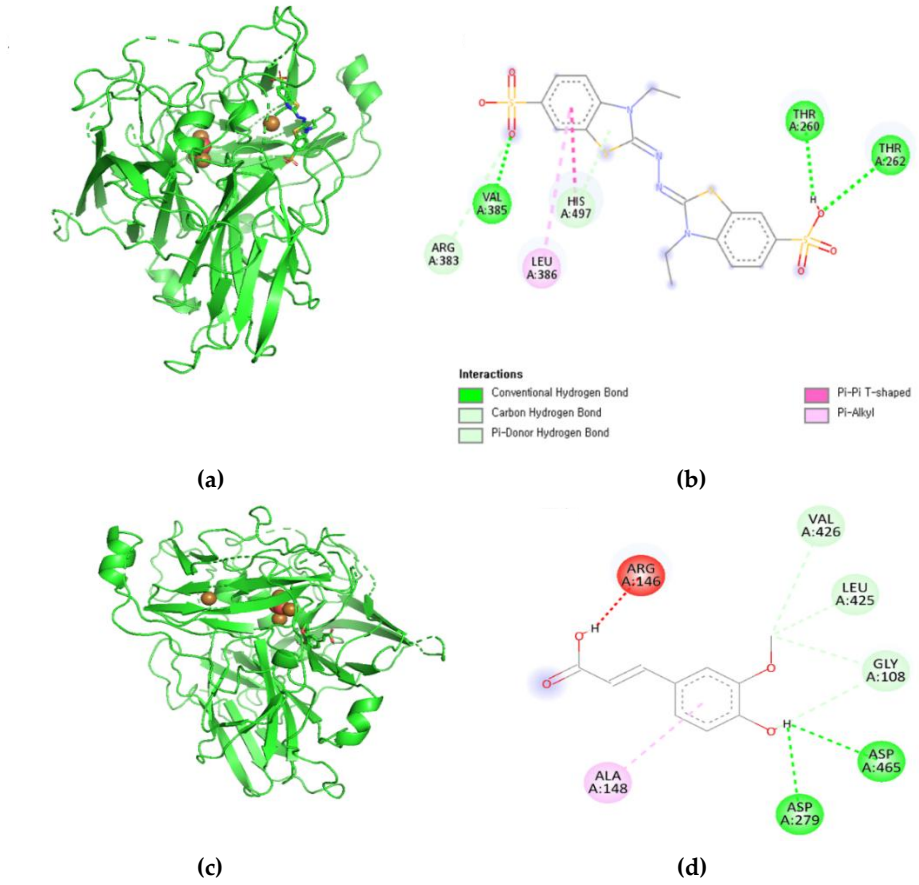

Figure 3. Laccase docked with artificial and natural mediators with interaction pose: (a) the docking complexed of laccase with ABTS; (b) the interaction poses with different resides of predicted laccase; (c) and (d) the docking and interaction posed of laccase with ferulic acid.

4. Discussion

Following Yoshida's discovery of laccase in Japanese lacquer, its vast potential and the onset of a green revolution were unforeseen [17]. Today, it's widely recognized among laccase researchers that natural and/or engineered laccases are central to the revolution. This current challenge lies in producing these enzymes in large quantities and tailoring their biotechnological properties through laboratory evolution combined with rational and semi-rational approaches. Laccase catalyzes two types of reactions: direct oxidation and indirect oxidation. Direct oxidation involves substrate oxidation to the corresponding radical due to direct interaction with the copper cluster [18]. However, in some cases, direct oxidation isn't possible as laccase can only oxidize compounds with an ionization potential below the redox potential of the T1 copper ion [19]. Nevertheless, this limitation can be circumvented using a mediator, a two-step process where the enzyme first oxidizes the mediator which then oxidizes the substrate.

Virtual screening methods are categorized into structure-based virtual screening (SBVS) and ligand-based virtual screening (LBVS). SBVS, which involves docking, requires a 3D structure of the protein target [20], while LBVS techniques utilize libraries of known ligands [21]. Among SBVS techniques, we utilized ChimeraX, which supports various stages of atomic model construction, including starting model generation, fitting, refinement, validation, and visualization [16]. Although AlphaFold structure prediction in ChimeraX provides initial protein models, their accuracy is not as high as experimental models [22]. These predictions, however, offer a close sequences match with high confidence, suitable as templates for conventional homology modelling [23]. Autodock Vina, frequently used as a reference for docking programs and scoring functions, was employed in our studies [24]. Docking experiments involving mediator-enzyme interactions indicate that artificial mediator molecules closely approach the predicted laccase compared to the natural mediators. Docking possesses also suggest proximity to the type T1 catalytic sites, as observed in the laccase-ABTS complex (Figs. 3 (a) and 3 (b)). Furthermore, in terms of binding affinity, natural mediators such as ferulic acid and coumaric acid exhibit a comparable interaction with the predicted laccase, like artificial mediators like N-hydroxyacetanilide and N-hydroxyphthalimide. These natural mediators are phenolic molecules derived from lignin, offering a sustainable and renewable alternative [25]. Additionally, they demonstrate stability in both their economic and environmentally friendly characteristics. Virtual screening hits represent compounds anticipated to interact with the target of interest, yet their efficacy requires experimental validation [14]. Laboratory assessment typically involves employing a commercial enzyme to detect enzyme activity and comparing the target's activity in the presence and absence of the identified hit compound.

5. Conclusions

In conclusion, this study argued that it is desirable to use some natural mediators such as coumaric acid and ferulic acid for laccase oxidation reduction reaction in place of artificial mediators. This kind of prediction approach could reduce the screening of many mediators for experimental validation which ultimately reduces the cost. It may be worth more if the docking could be done in target-specific scoring functions for receptors and binding affinities.

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