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Research article

Multiple ligand simultaneous docking (MLSD): A novel approach to study the effect of inhibitors on substrate binding to PPO

S. Raghavendra[a], S.J. Aditya Rao[b,*], Vadlapudi Kumar[c], C.K. Ramesh[b]

[a] College of Horticulture, University of Horticulture Sciences, Bagalkot 587102, Karnataka, India
[b] Molecular Biomedicine Laboratory, PG Department of Biotechnology, Sahyadri Science College, Shimoga 577203, Karnataka, India
[c] Department of Biochemistry, Davangere University, Shivagangothri, Davangere 577002, Karnataka, India

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ABSTRACT

Multiple ligand simultaneous docking, a computational approach is used to study the concurrent interactions between substrate and the macromolecule binding together in the presence of an inhibitor. The present investigation deals with the study of the effect of different inhibitors on binding of substrate to the protein Polyphenoloxidase (PPO). The protein was isolated from *Mucuna pruriens* and confirmed as tyrosinases involved in L-DOPA production. The activity was measured using different inhibitors at different concentrations taking catechol as substrate. A high-throughput binding study was conducted to compare the binding orientations of individual ligands and multiple ligands employing Autodock 4.2. The results of single substrate docking showed a better binding of urea with the binding energy of $-3.48\ \mathrm{kJ\,mol^{-1}}$ and inter molecular energy of $-3.48\ \mathrm{kJ\,mol^{-1}}$ while the results of MLSD revealed that ascorbic acid combined with the substrate showed better inhibition with a decreased binding energy of $-2.37\ \mathrm{kJ\,mol^{-1}}$.

1. Introduction

In this post-genomic era, progressively more research is focused on proteomics. Experimental and computational efforts are devoted to large-scale generation and analysis of information derived from 3D structures and dynamics of proteins, with the goal of scientific and commercial break-through in drug discovery. This raises the necessity for new and fast computational approaches alternative to wet-lab high-throughput screens for drug discovery (Maggio and Ramnaraya, 2001; Abagyan and Totrov, 2001; Klebe, 2000; Moitessier et al., 2008; Cheng et al., 2012; Halperin et al., 2002). A necessary prerequisite for successful differentiation between active and non-active ligands in the accurate prediction of their binding affinities to the complex is by use of docking score functions (Atkovska et al., 2014). In this regard, molecular docking has been extensively applied in effective screening of small molecule libraries for lead identification and optimization.

Polyphenoloxidases are copper containing metalloproteins that catalyzes the hydroxylation of o-monophenols to o-diphenols (E.C. 1.14.18.1; monophenolmono oxygenase, tyrosinase, cresolase) and the oxidation of o-dihydroxyphenols to o-quinones (E.C. 1.10.3.2; diphenol oxygen oxidoreductase, diphenol oxidase, or catecholase). The structure of the active site of the enzyme, in which copper is bound by six or seven histidine residues and a single cysteine residue is highly conserved. The enzyme seems to be of almost universal distribution in animals, plants, fungi and bacteria. PPO-mediated browning damage is one of the main causes of quality loss in fresh fruit and processed food (Chi et al., 2014). The importance of PPO in browning reactions continues to occupy many researchers and many subsequent publications describe browning reactions in a variety of species and their tissues. The consequent non-enzymatic polymerization of the quinines leads to formation of brown pigments that are the cause of post-harvest deterioration and loss of quality in many economically important crops (Mayer, 2006).

In the present study, an attempt was made on multiple ligand simultaneous docking (MLSD) on Polyphenol oxidase to mimic the real molecular recognition processes better. Docking multiple substrates simultaneously at the enzymatic sites of PPO would reflect the real biochemical process, gaining mechanistic insights of the concerted action of multiple substrates/cofactors when they approach the binding pockets. Binding dynamics, including possible intermediates and transition states, could even be revealed energetically (Li and Li, 2010).

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* Corresponding author.
E-mail address: adityaraosj@gmail.com (S.J. Aditya Rao).

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Table 1
Substrate specificity of PPO enzyme.

Substrate	Specific activity (unit mg^{-1} protein)	Km (μM)	Vmax (Abs min^{-1})
Catechol	2300	4.12×10^{-3}	3020
L-DOPA	430	16.40×10^{-3}	990
L-Tyrosine	1930	6.12×10^{-3}	2930
p-Cresol	2110	5.01×10^{-3}	2983

2. Materials and methods

2.1. Extraction, assay, characterization of PPO and substrate specificity

The protocol for extraction, assay and characterizations of PPO were driven from our earlier work (Raghavendra et al., 2011) for which the same is discussed in brief. Polyphenol oxidase (PPO EC 1.14.18.1) was isolated from *Mucuna pruriens* and confirmed as tyrosinases involved in L-DOPA production. The purified enzyme was confirmed as PPO by activity staining, and SDS-PAGE analysis. Substrate specificity experiments were carried out with catechol, L-DOPA, L-tyrosine, and p-cresol. Of these, catechol was evaluated as the most suitable substrate (Table 1). PPO activity was measured using different inhibitors at different concentrations (20 mM to 100 mM in a 0.1 M phosphate buffer with a pH of 7.0) using catechol as the substrate. In addition to these concentrations, the substances which showed better inhibition were further tested at 0.05 mM, 1 mM, and 2 mM concentrations (Table 2). Based on the results, L-ascorbic acid, citric acid, L-cysteine, and urea were selected for *in silico* studies.

2.2. In silico *studies*

2.2.1. Tools and servers used

Structures of drug molecules were drawn and analyzed using ChemDraw Ultra V6.0. The energy minimization was done using chemsketch v12.01. 3D coordinates were prepared using openbabel. The protein structural file (PDB ID: 2P3X) was fetched out from PDB (www.rcsb.org/pdb). Protein–ligand interactive visualization and analysis was carried out in Pymol viewer 1.5.4. ADME-T properties of molecules were identified using Organic chemistry portal (http://www.organic-chemistry.org/prog), a web-basedapplication for predicting *in-silico* ADME-T property. Molecular docking was performed using AutoDock v4.2 on Windows 7 plat form (64-bit) with Lenovo G570 machine (Intel Core i5-560 M Processor 2.66 GHz, 4 GB memory).

2.2.2. Preparation of inhibitors

Structure of the substrate and inhibitor molecules were drawn in ChemDraw Ultra 6.0 assigned with proper 2D orientation (ChemOffice package) and the structure of each molecule was analyzed using Chem-3D Ultra 6.0 (ChemOffice package). 3-D geometrical optimization was done using Chemsketch v12.01 (ACD/labs). Openbabel, a standalone tool was used to obtain 3D coordinates of respective molecules.

Table 2
Effect of inhibitors on PPO enzyme activity.

Inhibitors conc. in mM	% Inhibition						
	0.05	1.0	2.0	20	40	80	100
Ascorbic acid	100	100	100	100	100	100	100
Citric acid	100	100	100	100	100	100	100
Cysteine	80	90	100	100	100	100	100
Urea	00	00	00	00	20	60	80

2.2.3. In-silico *ADME-toxicology prediction*

Organic chemistry portal (http://www.organic-chemistry.org/prog), a web-based application was used for prediction of ADME-T properties. The selected molecules were assessed for Pharmacodynamic parameters like mutagenicity, tumerogenicity, reproductive effect and ocular & skin irritancy and pharmacokinetic properties like solubility, human intestinal absorption and blood brain barrier permeability. Based on the results, each ligand was assigned with respective drug-likeness and drug score.

2.2.4. Preparation of macromolecule

Polyphenol oxidase (tyrosinase) (TY) is a bifunctional, copper-containing oxidase having both catecholase and cresolaseactivity. The crystal structure of polyphenol oxidase (Fig. 1) was isolated using Cu_2O as ligand molecule (Malstrom and Ryden, 1968). The protein structure file (PDB ID: 2P3X) was retrieved from PDB (www.rcsb.org/pdb) and is edited by removing the hetero atoms like metal ions, water and ligand molecule. Later it was added with C-terminal oxygen, polar hydrogen, and Gasteiger charges.

2.2.5. Single ligand docking

Automated docking was used to study the binding of different drug molecules to the active pocket of polyphenol oxidase. AutoDock uses a semi-empirical free energy force field as the scoring function to evaluate the docked conformation solution (Morris et al., 1998). The scoring function includes the pair-wise evaluations and entropy change upon binding (Huey et al., 2007). A genetic algorithm method implemented in the AutoDock 4.2 was employed to study appropriate binding modes of the ligand in different conformations. For the ligand molecules, Gasteigere–Marsili partial charges were assigned and non-polar hydrogen atoms were merged (Gasteiger and Marsili, 1980). All torsions were allowed to rotate during docking. The structure of the active pocket

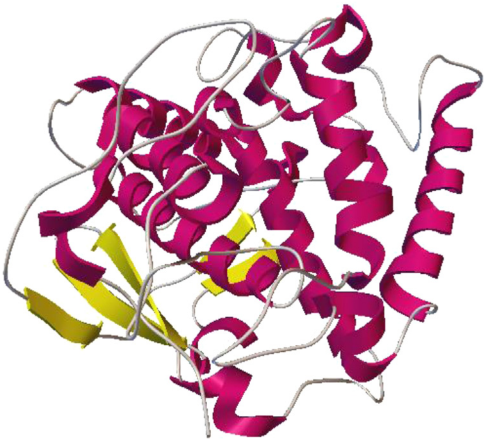

Fig. 1. Structure of polyphenol oxidase (PDB ID: 2P3X).