Chapter IV

Substrate Specificity and Active Site Comparison

4.1 Introduction [52]

Molecular docking is the method to evaluate the feasible binding geometries of a putative ligand with a protein target in the three dimensional view. The binding geometries, often called binding modes or poses include, in principle, both positioning of the ligand relative to the receptor and conformational states of the ligand and the receptor. Docking method can be evaluated by their ability to rapidly and accurately dock large numbers of small molecules into the binding site of receptors. Therefore, the essential feature of any ligand-receptor interaction is the correct estimation of free energy of binding.

There are three basic tasks any docking procedure must accomplish:

1. Characterization of the binding site
2. Positioning of the ligand into the binding site
3. Evaluating the strength of interaction for a speciflc ligand-receptor complex

The study of substrate docking in the active center of *Arthromyces ramosus* peroxidase (ARP) focuses on the phenol derivative substances. The lowest docking energies of 1-naphthol were found for pentamer (-13.3kcal/mol), of 2-naphthol were found for pentamer (-13.9kcal/mol) and of 4-hydroxybiphenyl were found for trimer

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(-12.4kcal/mol). The calculated docking energies of phenol derivatives showed that the higher molecular weight of oligomer examined had the higher affinity to ARP. The lowest average docking energy was reported for trimer of 4-hydroxybiphenyl for ARP [53, 54]. The docking of sweet potato catechol oxidase (ibCO) using 4-tertiary butylcatechol has the best docking energy of -7.3 kcal/mol.

4.2 Methodology

4.2.1 Structure preparation

Using the established grape ascorbate peroxidase homology model or the x-ray structure of grape polyphenol oxidase (PDB ID: 2P3X) as the receptor. To prepare the crystal structure of polyphenol oxidase, the protein was purified from Grenache grape berries by using traditional methods and crystallized with ammonium acetate by the hanging-drop vapor diffusion method. The structure was obtained at 2.2 Å resolution. Energy minimization was performed by using 1,000 steps of steepest descent method.

Schematic representations of the ligand; for each of enzymes used in this study are shown in Figure 4.1. The 3D structures of ligands were sketched and optimized with AM1 method.

4.2.2 Docking study

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A CHARMm-based docking program CDOCKER algorithm was employed to find the potential binding mode between both enzymes and the phenolic compound ligand. The active site pocket of the receptor was found automatically by the Discovery Studio1.7. The site sphere radius of 14 Å of grape peroxidase and 7 Å of

grape polyphenol oxidase were set to assign the entire ligand binding pocket. In CDOCKER, random ligand conformations are generated through molecular dynamics, and a variable number of rigid-body rotations/translations are applied to each conformation to generate the initial ligand poses. The conformations are further refined by grid-based simulated annealing in the receptor active site, which makes the results accurate. The CDOCKER interaction energy between the substrates/inhibitors to enzymes was finally computed. The complex structure with the lowest interaction energy was used for comparison.

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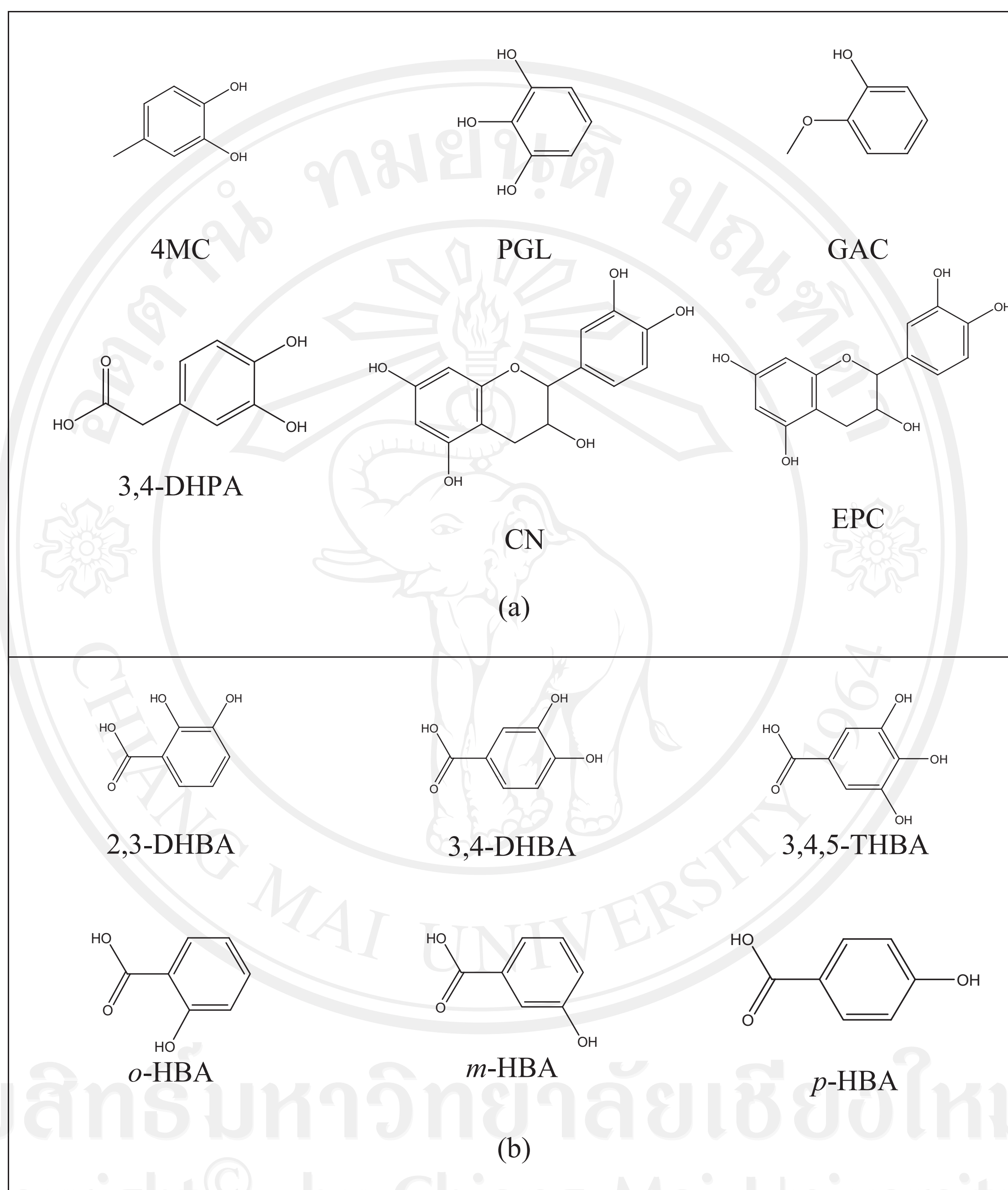

Abbreviations
CAT, catechol; 4MC, 4-methylcatechol; PGL, pyrogallol; GAC, guaiacol; 3,4-DHPA, 3,4-dihydroxyphenylacetic acid; CN, catechin; EPC, epicatechin; 2,3-DHBA, 2,3-dihydroxybenzoic acid; 3,4-DHBA, 3,4-dihydroxybenzoic acid; 3,4,5-THBA, 3,4,5-trihydroxybenzoic acid; *o*-HBA, *o*-hydroxybenzoic acid; *m*-HBA, *m*-hydroxybenzoic acid; *p*-HBA, *p*-hydroxybenzoic acid

Figure 4.1 Chemical structures of selected substrates (a) and inhibitors (b) for docking experiment.

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4.3 Results and discussion

4.3.1 Comparison of substrate binding site for PPO and POD from molecular docking

The docking of grape peroxidase and polyphenol oxidase with each substrate, 4-methylcatechol, guaiacol, pyrogallol, 3,4-dihydroxyphenyl acetic acid, catechin and epicatechin , were calculated. The conformation with the lowest binding interaction energy was selected. From our models, PPO has slightly smaller binding pocket than POD. Therefore, the number of binding amino acid residues has been observed. All the residues with less than 3Å distances to epicatechin are represented in Figure 3, including His87, Phe113, Asn240, His243, Lys244, Gly257, Phe259, Ala262, Phe268 for grape polyphenol oxidase and Arg37, Ala69, Asn71, Leu130, Pro131, Asn132, Ala133, Ser171 for ascorbate peroxidase of grape. For comparison, the energies obtained from docking of each ligand are listed in Table 2. The more negative interaction energy exhibit the more favorable binding. The prediction of interaction energy with the substrates and inhibitors of grape peroxidase are generally lower than that of grape polyphenol oxidase. The substrates with high affinity were epicatechin and catechin with -45.63 and -44.75 kcal/mol for peroxidase and -42.99 and -45.55 kcal/mol for polyphenol oxidase, respectively. Other complex did not show the difference in binding affinity according to the interaction energy range from -25.91 to -35.46 kcal/mol for peroxidase and -23.93 to -53.55 kcal/mol for polyphenol oxidase. The selected ligands frequently form hydrogen bonds with Gly257 of grape polyphenol oxidase and Arg37 of grape ascorbate peroxidase. A hydrogen bond

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interactions were determined using the following criteria: (i) the distance between proton donor (D) and acceptor (A) atoms ≤ 3.5 Å and (ii) the D-H...A angle = 120Ǻ. Similary, Tatoli *et.al.* had reported the strong hydrogen bonding between Arg38 side chain and peroxy-complex of recombinant horseradish peroxidase, which is one of the most studied enzymes among the heme peroxidases for its importance in modern enzymology [55]. A commonly accepted mechanism for peroxidases proposed many years ago by Poulos-Kraut [56] has also reported the important of the highly conserved His42 and Arg38 residues in the stepwise acid-base catalysis.

4.3.2 Specificity of inhibitors for PPO and POD: Theoretical and experimental comparison

From experimental studies, various potent inhibitors for grape polyphenol oxidase were ascorbic acid, cysteine, and sodium metabissulfite [57] whereas cysteine inhibited polyphenol oxidase activity in mango puree [58] and was effective in preventing the browning of apple juice [18, 24]. However, cysteine produces an undesirable order, limiting its use in food processing. The aromatic carboxylic acids (benzoic and cinnamic acid) were inhibitors, due to their structural similarity with phenolic substrates [59]. In order to study the binding mode of the inhibitors, benzoic acid and its analogs shown to control enzymatic browning, were chosen for the investigation. The calculated docking interaction energy of benzoic compound showed high affinity to grape ascorbate peroxidase and polyphenol oxidase (Table 4.1). Ferrer and coworker reported that 2,3-dihydroxybenzoic acid showed no

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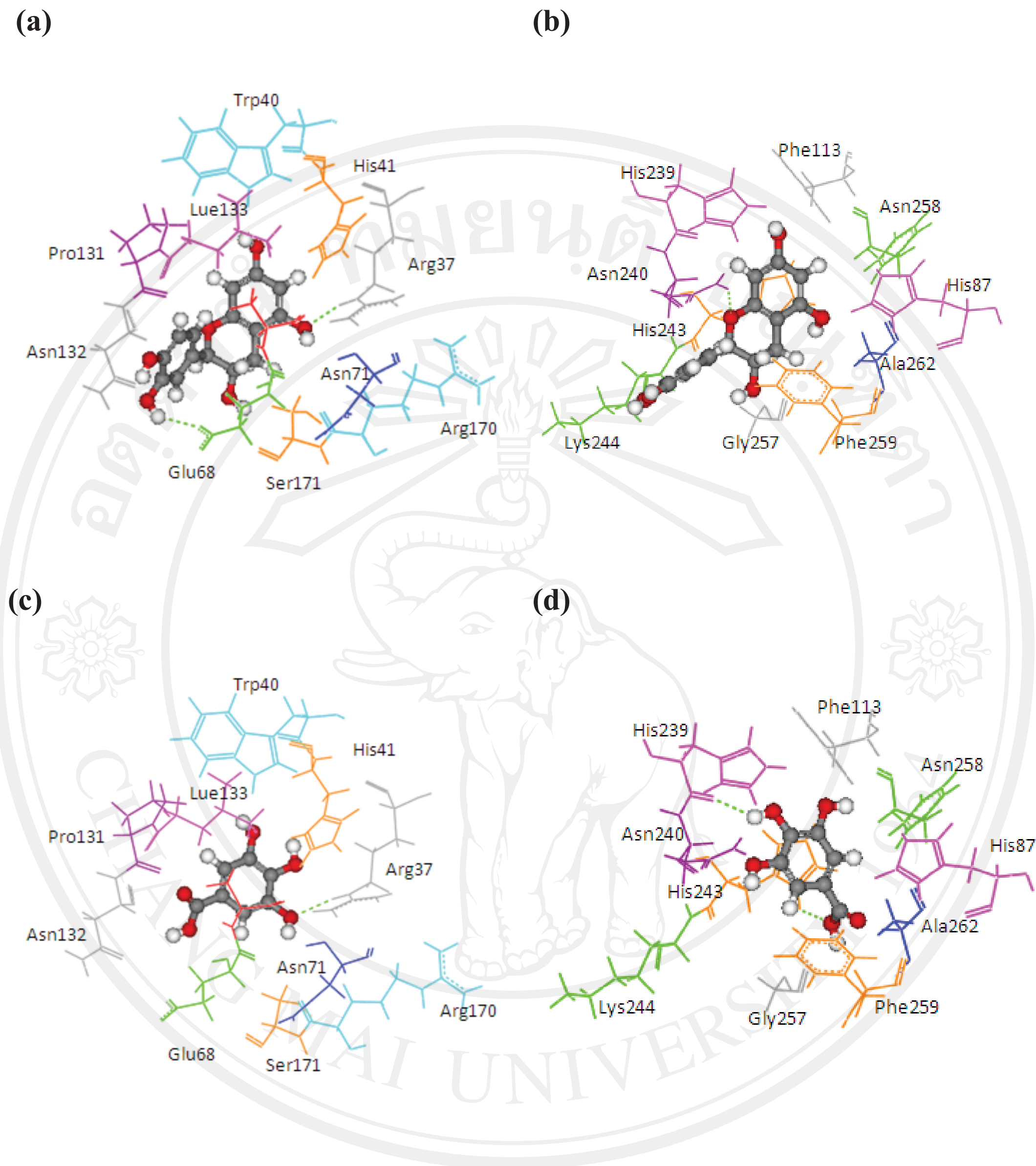

Figure 4.2 3Å binding site comparison of PPO and POD with common substrate and inhibitor (as in ball and stick model). Dashed line represents H-bond. (a) POD with EPC (b) PPO with EPC (c) POD with 3,4,5-THBA (d) PPO with 3,4,5-THBA

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Table 4.1 Experimental predicted interaction of phenolic and benzoic acid compounds with grape ascorbate peroxidase and polyphenol oxidase

		ABX(POD)				2P3X(PPO)			
Substrate	**Structure**	**Experimental value [60] $K_m(x10^{-3}M)$**	**Interaction energy (kcal/mol)**	**No. of hydrogen bonding**	**Residue in hydrogen bonding**	**Relative activity[61]**	**Interactio n energy (kcal/mol)**	**No. of hydrogen bonding**	**Residue in hydrogen bonding**
Substrates									
4MC		22.0	-28.23	1	Arg37	100	-41.85	1	His239
GAC		32.2	-28.49	2	Arg37		-23.93	0	
PGL		32.2	-30.45	2	Arg37	78.1	-28.78	0	
3,4-DHPA		na	-35.46	2	Trp40 Arg170	na	-53.55	2	His239 Gly257
CN		5.2	-44.75	2	Arg37 Glu68	na	-45.55	2	Asn240 Gly257
EPC		5.2	-45.63	2	Arg37 Glu68	93.1	-42.99	1	Asn240
Inhibitors									
2,3-DHBA		na	-32.15	1	Pro131	na	-37.37	1	Gly257
3,4- DHBA		na	-31.38	1	Arg170	na	-44.71	1	His239
3,4,5- THBA		na	-34.76	1	Arg37	na	-43.01	4	His239 His243 Gly257 Asn258
o-HBA		na	-29.14	1	Arg37	na	-33.99	1	His239
m-HBA		na	-29.17	0		na	-39.04	1	Gly257
p-HBA		na	-26.23	1	Trp40	na	-36.68	2	Glu235 Gly257

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inhibitory effect whereas 2,4-dihydroxybenzoic acid was a strong polyphenol oxidase inhibitor [62]. From our docking study, the inhibitors 3,4,5-trihydroxybenzoic acid has high affinity with both enzymes. The series of monohydroxybenzoic acids (*m-, o-, p*-hydroxybenzoic acid) have high affinities with grape polyphenol oxidase with lower negative interaction energy values than those with peroxidase. Other compounds including 2,3-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, o-hydroxybenzoic acid, m-hydroxybenzoic acid, can be used as common inhibitors for both enzymes.

4.3.3 Future applications

These results suggest that the apparent positions of key groups (carboxylic and hydroxyl) are important in the investigation of either substrate or inhibitor activity. Compounds which have same basic phenyl ring, were successfully docked at the active site of polyphenol oxidase and peroxidase. The positions of the hydroxyl, carboxyl groups and the bulkiness and length of the side chain lead to different interactions between the active site and the inhibitor. Benzoic acid and its sodium salt have long been used to control enzymatic browning. In addition, sulfur dioxide and its derivatives have also been applied in food industry. The food and drug administration is re-evaluating the use of chemical agents as food additives and their use in some products is banned. A study of the naturally occurring, highly active inhibitors of tyrosinase, the chalcones and related compounds, showed that the number and position of hydroxyl groups were important to the degree of inhibition. Experimental works may be carried out to support the findings from this study. Common inhibitors predicted by molecular modeling can be tested for their abilities to inhibit browing

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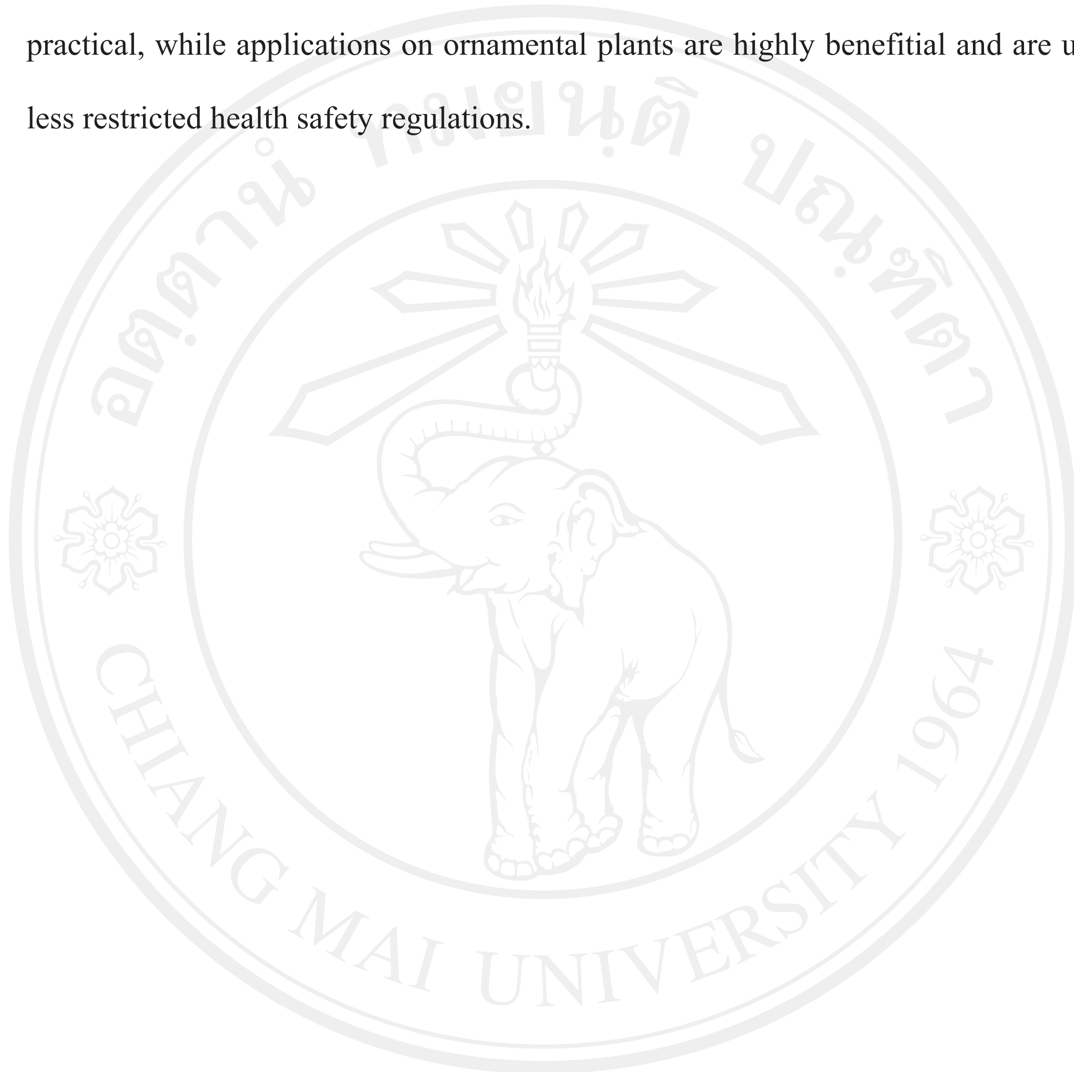
caused by both polyphenol oxidase and peroxidase in grapes and other plants of interest. Applications on edible plants for commercial use need to be safe and practical, while applications on ornamental plants are highly benefitial and are under less restricted health safety regulations.



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