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Inhibition of mushroom tyrosinase by phenol derivative (HDNOS) ligand

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Abstract

The inhibitory effect of 2-{1-(E)-2-((Z)-2- {(E)-2- [(Z)-1-(2hydroxyphenyl) ethylidene] hydrazono}-1-methylpropylidene)hydrazon o]ethyl}phenol (HDNOS) on both activities of mushroom tyrosinase (MT) at 20°C in a 10 mM phosphate buffer solution (pH 6.8), was studied. L-Dopa and L-Tyrosine were used as substrates of catecholase and cresolase activities, respectively. The results showed that ligand competitively inhibits both activities of the enzyme with inhibition constants (K_i) of 5.26 ± 0.1 and 4.76 ± 0.1μM for catecholase and cresolase respectively, which are good valuses comparing with the reported values for other MT inhibitors. For further insight a docking study between tyrosinase and ligand was performed. The docking simulation showed that ligand binds in the active site of the enzyme near the Cu atoms.

Key words: Mushroom Tyrosinase, Inhibition, cresolase, catecholase

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1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper containing enzyme that is widely distributed in microorganisms, animals, and plants [1-3]. The enzyme catalyzes two different reactions: 1- cresolase activity (monophenolase activity) that hydroxylates monophenol (L-tyrosine) to diphenol (L-Dopa) and catecholase activity (diphenolase activity) that oxidizes diphenol (L-Dopa) to o-quinone [4].

The enzymatic oxidation of L-tyrosine to melanin is very important because melanin has many functions, and alterations in the synthesis of melanin contribute to some diseases [5]. Finding new tyrosinase inhibitors with low Ki values is very important because tyrosinase has a major role in both mammalian melanogenesis and enzymatic browning of fruit or fungi. In melanogenesis some pigments, like melanin, will be produced. Melanin is formed through the combination of enzymatically catalyzed and chemical reactions. Raper [6] and Mason [7] have elucidated the biosynthetic pathway of the formation of melanin, and Cooksey et al. [8] and Schallreuteret al. [9] modified this pathway. In the melanogenesis, first tyrosinase oxidizes tyrosine to dopaquinone; a reaction which is the rate-determining step in the synthesis of melanin since the remainder of the reaction sequence can spontaneously proceed at the physiological pH values[10]. Melanin protects human skin from the harmful effects of UV from the sun [11]. Since hyperpigmentation in human skin is not desirable, researchers are interested in finding new potent tyrosinase inhibitors for the use in anti-browning and skin whitening. The active site of tyrosinase has a di-copper center that is similar but not identical to hemocyanins [12, 13]. Each copper ion in the active site is coordinated with three nitrogen atoms from the three adjacent histidine residues [14, 15]. Mushroom tyrosinase (MT) is the commercially available tyrosinase. The control of tyrosinase activity has been of special interest among investigators due to its potential use in medicinal, cosmetic, and agricultural products [16]. Therefore, understanding the catalysis mechanism of tyrosinase and its regulation including inhibition is important. To understand the mechanism of the enzymatic and inhibition reactions, many studies have been performed to obtain additional information about the function of MT [16-21]. After introducing two new bi-pyridine synthetic compounds as potent uncompetitive MT inhibitors [21], the inhibitory effects of three synthetic n-alkyl dithiocarbamates [22], n-alkyl xanthates [23, 24], and

benzyl and *p*-xylidine-bisdithiocarbamate [25] were elucidated. The inhibitory effects of isophthalic acid, terephthalic acid, phthalic acid, trifluoroethanol and chlorophenolson on mushroom tyrosinase have also been studied recently [26-30]. Different kinetic mechanisms for the function of tyrosinase have been suggested so far [31, 32]. The enzymatic reaction mechanism consists of two parts namely cresolase and catecholase activities. At first an oxygen molecule binds to E_d (deoxy form of the enzyme) converting it to E_o (oxy form of the enzyme). The produced E_o can participate in two different reactions by binding to monophenol or diphenol substrates. In case of the former reaction an E_oM complex (oxy form with monophenol) will form, which finally converts to an E_mD complex (met form of the enzyme with diphenol). At this point the enzyme oxidize bound D (diphenol) to *o*-quinone (Q), producing *deoxy*-tyrosinase (E_d). It is also proposed that Q involves spontaneous intramolecular cyclization and further redox steps and converts to dopachrome (DC) and D. The D produced in this reaction involves in the transformation of the E_m form of the enzyme (which is inactive towards M) into the E_o form (which is active towards M) giving rise to a lag time, which is a characteristic of the cresolase activity. If the E_o binds to catechol substrate, the E_oD complex will form, which converts to E_m through the oxidation of catechol to quinone. E_m can bind to both substrates and binding to the catechol substrate can oxidize it to quinone, but if it binds to the monophenol substrate, a dead end complex (E_mM) will form which contributes to the lag phase in cresolase activity [33-35]. Tyrosinase molecule contains two copper atoms, and each atom of the binuclear copper cluster is ligated to three histidines. In the formation of melanin pigments, three types of tyrosinase (oxy-, met-, and deoxy tyrosinase), are involved each having a different binuclear copper structures in the active site. The oxygenated form (oxy tyrosinase, E_o) consists of two tetragonal copper (II) atoms, each coordinated by two strong equatorial and one weaker axial N_{His} ligand. The exogenous oxygen molecule is present as peroxide and bridges the two copper centers. Met tyrosinase (E_m), similar to the oxy form, contains two tetragonal copper (II) ions coupled through an endogenous bridge, although hydroxide exogenous ligands other than peroxide are bound to the copper site. Deoxy tyrosinase (E_d) contains two copper (I) ions with a co-ordination arrangement similar to that of the met form, but lacking hydroxide bridge. The resting form of tyrosinase, i.e., the enzyme as obtained after purification, is found to be a mixture of 85% met and 15% oxy forms. MT is active and performs the cresolase and catecholase reactions when its Cu atoms are in Cu^{+2} forms. So it is expected that by blocking the Cu^{+2} in the active site the enzyme activity would be inhibited. Recently the three dimensional structure of mushroom tyrosinase has been determined [36, 37]. For gaining more insights on the binding model, a docking simulation was performed. As mentioned above, the Cu atoms in the active site are very important for catalytic activity of the enzyme so designing new inhibitors that can tightly bind to Cu atoms, can lead to inhibition of the enzyme activity. Many inhibitors including different dioles, monoamines and diamines that can bind to the Cu atoms in the active site have been designed so far [38-42].

The results of these studies revealed that hesperetin inhibits tyrosinase activity through forming hydrogen bonds with histidine residues. This synthesized ligand (Fig. 1) has two hydroxyl groups and also has high tendency to copper ions [43]. So it is expected that this ligand makes complex with copper ions in the MT active site and blocks the entrance of substrates to it.

2. Materials and Methods

2.1. Materials

Mushroom tyrosinase (MT; EC 1.14.18.1), specific activity 5340 units/mg, L-DOPA and L-tyrosine were purchased from Sigma Co. Phosphate buffer (10 mM, pH 6.8) was used throughout this research and the corresponding salts were obtained from Merck Co. All experiments were carried out at 20°C.

2.2. Methods

2.2.1. Synthesis of ligand

Synthesis of the ligand was performed according to previous method [43].

2.2.2. Kinetic measurements

Catecholase and cresolase activities of MT were determined in the reaction medium for 1 and 2 min, with enzyme concentrations of 11.11 and 112.68 µg/ml by spectrophotometrically measuring the formation rate of dopachrome at 475 nm (ε= 3700 M^{-1} cm^{-1}) in the first two minutes using a Cary spectrophotometer, 100 Biomodel, with jacketed cell holders. The assay was performed as previously described with slight modifications [44]. Freshly dissolved enzyme, substrate and ligand were used in this work. All enzymatic reactions were performed in a phosphate buffer (10 mM) at pH 6.8 in a conventional thermostated quartz cell. Substrate addition followed after incubation of enzyme with different concentrations of ligand. Final substrates concentrations for catechol (L-Dopa) and cresole (L-tyrosine) were 100 µM. The initial rate was measured in each assay, and repeated three times.

2.2.3. In Silico Docking of the Tyrosinase Structure and ligands

Because the crystallographic structure of tyrosinase has been recently depicted a template structure from the PDB was selected to simulate the docking of ligand to tyrosinase. The minimized 3D tyrosinase was the input structure of HEX 5.1 protein docking software as the receptor. A blind docking was run with pre-generated ligand structures. The docking processes were performed under

shape and electrostatic correlation types in HEX [45]. A set of docking result was generated by HEX and the complex with the lowest energy was chosen as the best one. The post processing refinements were Newton like minimizations based on OPLS force field parameters. The last resulted structure was saved as a pdb file.

3. Results

The effect of ligand on both activities of MT was examined at pH 6.8 and temperature of 20°C.

3.1. Kinetic parameters of cresolase activity of MT in the presence of ligand

The effect of ligand on cresolase activity of MT was examined. Ligand was found to inhibit cresolase activity. The double reciprocal Lineweaver-Burk plot for the cresolase activity of MT assayed as hydroxylation of L-tyrosine, in the presence of different fixed concentrations of ligand are shown in Fig. 2. This plot shows a set of straight lines, which intersect exactly on the vertical axis; the value of maximum velocity (V_{max}) was unchanged by the inhibitor but the K_m' values were increased, which indicates competitive inhibition for ligand. Fig 3 shows the secondary plot, the K_m' at given concentration of inhibitor *versus* the concentration of inhibitor, which gives the inhibition constants (K_i) from the abscissa-intercepts ($-K_i$). The K_i value for ligand was 4.76 ± 0.1 μM the small value of showed that this ligand strongly bound to the enzyme.

3.2. Kinetic parameters of catecholase activity of MT in the presence of ligand

Double reciprocal Lineweaver-Burk plot for the catecholase activity of MT assayed as oxidation of L-DOPA in the presence of different fixed concentrations of ligand is shown in Fig. 4. This plot gives a set of straight lines intersecting exactly on the vertical axis; the value of maximum velocity (V_{max}) was unchanged by the inhibitor but the K_m' values were increased, which indicates competitive inhibition for ligand. Fig 5 shows the secondary plot, the K_m' at given concentration of inhibitor *versus* the concentration of inhibitor, which give the inhibition constants ($-K_i$) from the abscissa-intercepts. The K_i value for ligand was 5.26 ± 0.1 μM. So, it can be concluded that Ligand binds to the substrate-binding site and inhibits the catecholase activity in the competitive mode. The K_i value of ligand showed that this ligand strongly bound to enzyme. Docking result showed the binding site of ligand.

3.3. Ligand Docking Simulation

Docking result of ligand with the enzyme is shown in Fig 6. As the result shows, ligand is bound in the active site of MT.

4. Discussion

As previously mentioned during of the all assays, the ligand was first incubated with mushroom tyrosinase and then substrate was added to reaction medium. The initial rates were also measured so the probable effect of ligand on quinone could be ignored. As the experimental data show, ligand inhibits the enzyme competitively, which means it binds to the active site of the enzyme, which is further confirmed by docking results (Fig. 6). Ligand can form stable complexes with Cu^{+2}, so it blocks all enzyme forms that their oxidation state of the copper ions are in the Cu^{+2} form and the results confirm our proposal. By comparing the K_i values of different inhibitors we found out that ligand is a powerful inhibitor comparing to the previous inhibitors, which is shown in table 1 (see below). In this table, the K_i values of different potent inhibitors of the mushroom tyrosinase that can chelate the Cu atoms in the active site are compared (see below) the diamine and diol compounds are listed too (see below). As it is shown ligand had the small K_i value. As the docking result show, ligand is bound to Cu atoms. So we could synthesize ligand derivatives with smaller K_i to inhibit the tyrosinase and treat hyperpigmentation. Tyrosinases shares similar binuclear copper sites, so we could use MT as a model for human tyrosinase so it was probable that mushroom tyrosinase competitive inhibitors could inhibit human tyrosinase too. Also it has been found that aureusidin synthase (AUS) that has a key role in the yellow coloration of snapdragon flowers, has homology structure with plant polyphenol oxidase (PPO) [1], so probably ligand will inhibits AUS too because their active sites are identical.

The Cu atoms in the active site have the main role in tyrosinase catalysis. They cause these reactions through electron transfer to the substrates. When ligand formed a complex with Cu^{+2}, the electron transfer of Cu^{+2} changed so it could not perform the oxidation reactions with substrates and the enzyme would be inhibited.

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Table 1. Comparing K_i values of different inhibitors of MT for both cresolase and catecholase activities.

Reaction Type	Ligands	K_i (µM)	Reference
resolase activity	Ligand	4.76 ± 0.1	[24]
	$C_2H_5OCS_2Na$	13.8	[24]
	$C_3H_7OCS_2Na$	11	[24]
	$C_4H_9OCS_2Na$	8	[24]
	$C_6H_{13}OCS_2Na$	5	[23]
	Iso-propyl xanthate	9.8	[23]
	Iso-butyl xanthate	7.2	[24]
	Iso-pentylxanthate	6.1	[23]
	$C_4H_9NHCS_2Na$	0.8	[22]
	$C_6H_{13}NHCS_2Na$	1	[22]
	$C_8H_{17}NHCS_2Na$	1.8	[22]
	Norartocarpetin	1.354	[42]
	3-Amino-L-tyrosine	6.3	[44]
	2-aminophenol	0.9	[44]
Catecholase activity	Ligand	5.26 ± 0.1	-
	$C_2H_5OCS_2Na$	1.4	[24]
	$C_3H_7OCS_2Na$	5	[24]
	$C_4H_9OCS_2Na$	13	[24]
	$C_6H_{13}OCS_2Na$	25	[24]
	Iso-propyl xanthate	12.9	[23]
	Iso-butyl xanthate	21.8	[23]
	Iso-pentylxanthate	42.2	[23]
	$C_4H_9NHCS_2Na$	9.4	[22]
	$C_6H_{13}NHCS_2Na$	14.5	[22]
	$C_8H_{17}NHCS_2Na$	28.1	[22]
	Quercetin	29	[40]
	Galangin	58	[40]
	Fisetin	75	[40]
	3,7,4′_Trihydroxyflavone	154	[40]
	Morin	410	[40]
	Nobiletin	2820	[41]
	Hesperidin	9160	[41]
	Oxyresveratrol	0.91	[43]
	Hesperetin	4030	[45]

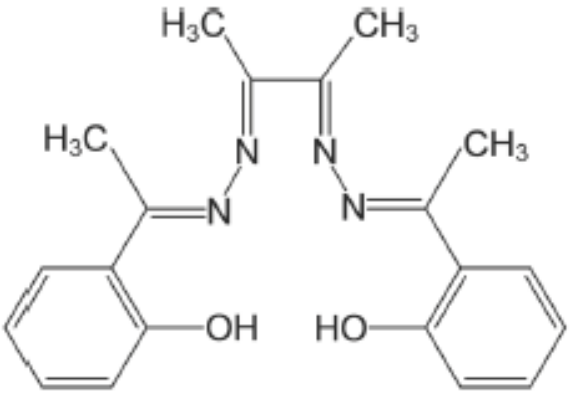

Fig.1. Chemical **s**tructure of the 2-{1-(E)-2-((Z)-2- {(E)-2- [(Z)-1-(2hydroxyphenyl) ethylidene] hydrazono}-1-methylpropylidene)hydrazon o]ethyl}phenol (HDNOS)

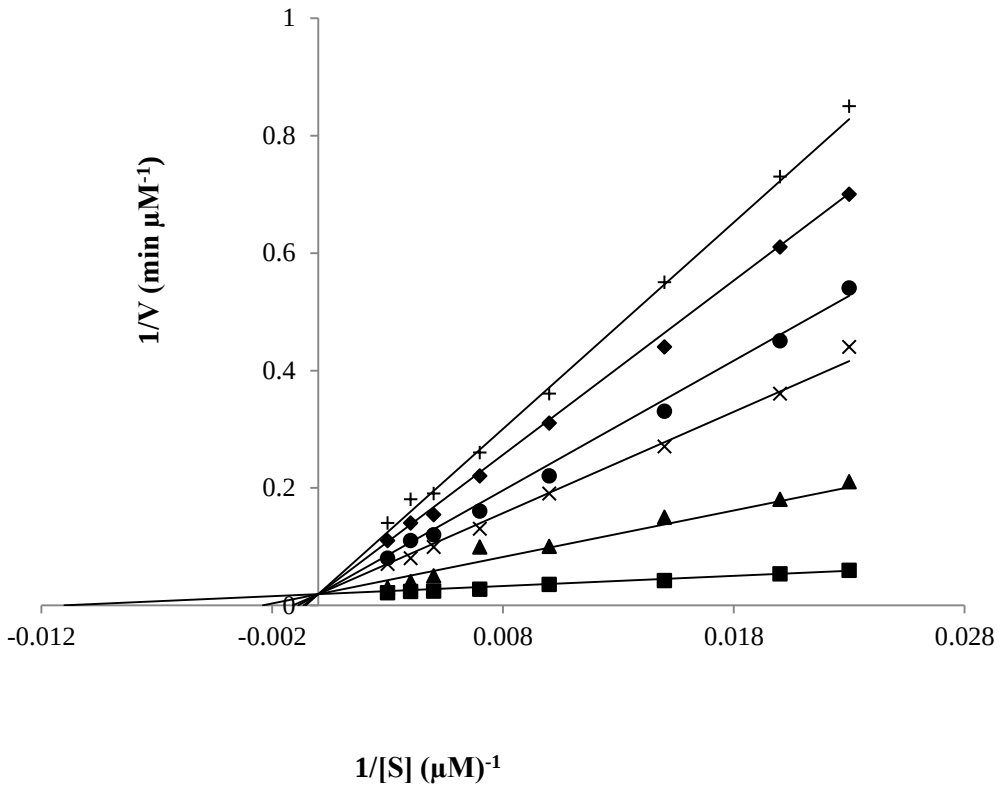

Fig. 2. Double reciprocal Lineweaver-Burk plot of MT kinetics assays for cresolase reactions of L-Tyrosine in 10 mM phosphate buffer, pH 6.8, at temperature of 20ºC and 112.68 µg/ml enzyme concentration, in the presence of different concentrations of ligand 0 µM (■), 0.7µM (▲), 0.9µM (×), 1.1 µM (●),1.3µM (♦), 1.5µM (+).

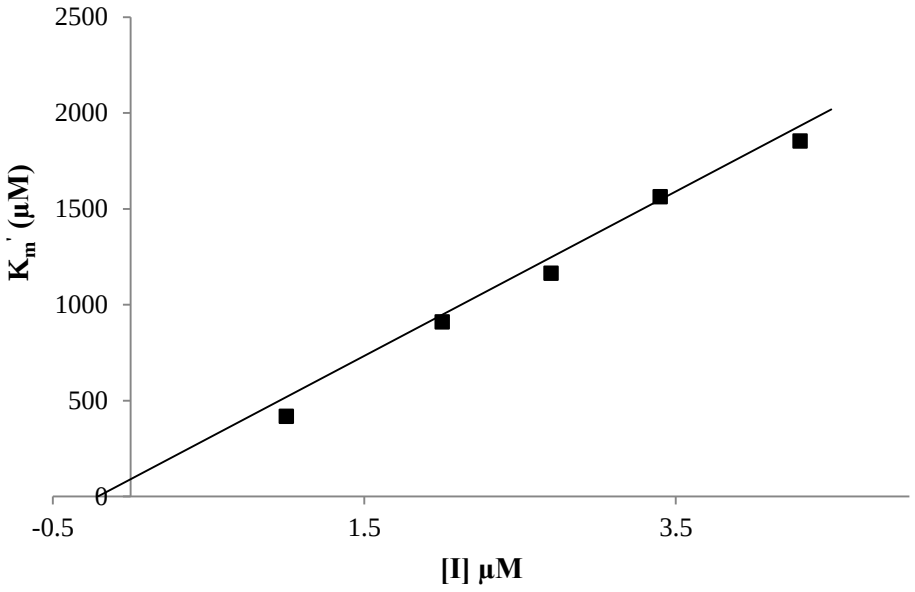

Fig. 3. Secondary plot for the cresolase activity, the K_m' at given concentration of ligand.*versus* the concentration of ligand.

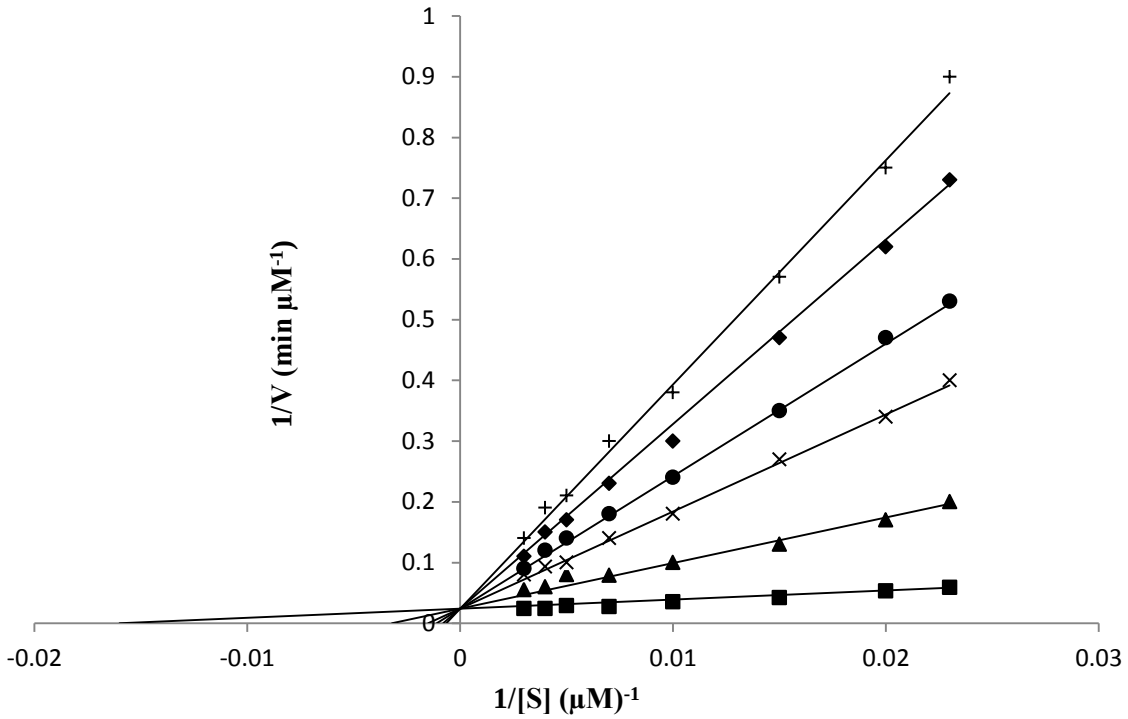

Fig. 4. Double reciprocal Lineweaver-Burk plot of MT kinetic assay for catecholase reactions of L-DOPA in 10 mM phosphate buffer, pH 6.8, at temperature of 20ºC and 11.11 µg/ml enzyme concentration, in the presence of different concentrations of ligand: 0 µM (■),0.7µM (▲), 0.9µM (×), 1.1 µM (●),1.3 µM (♦), 1.5 µM (+).

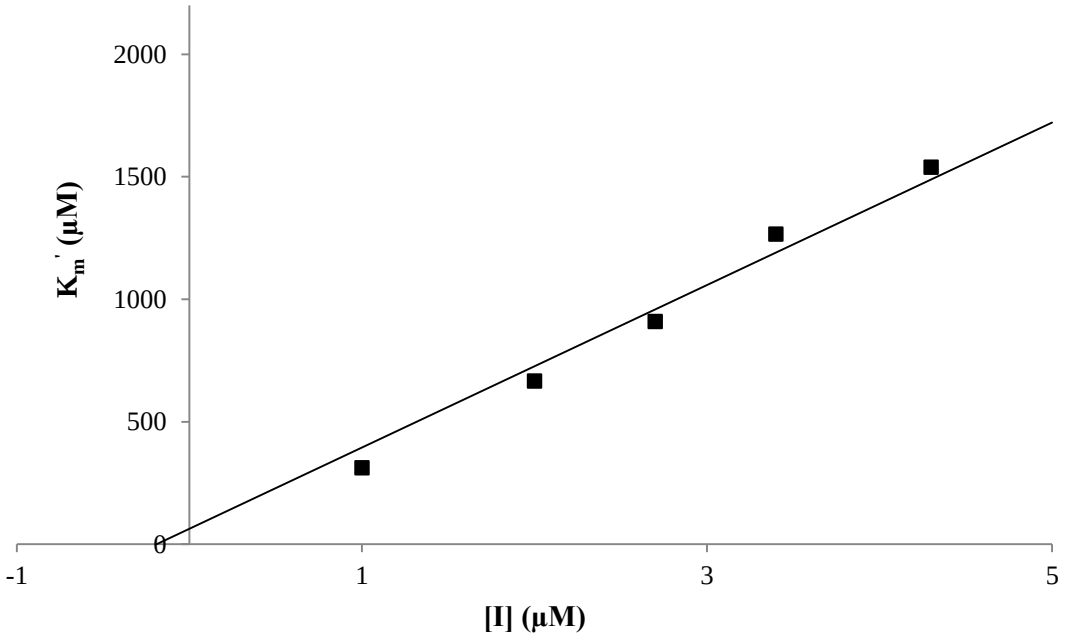

Fig. 5. Secondary plot for the catecholase activity, the K_m' at given concentration of ligand. *versus* the concentration of ligand.

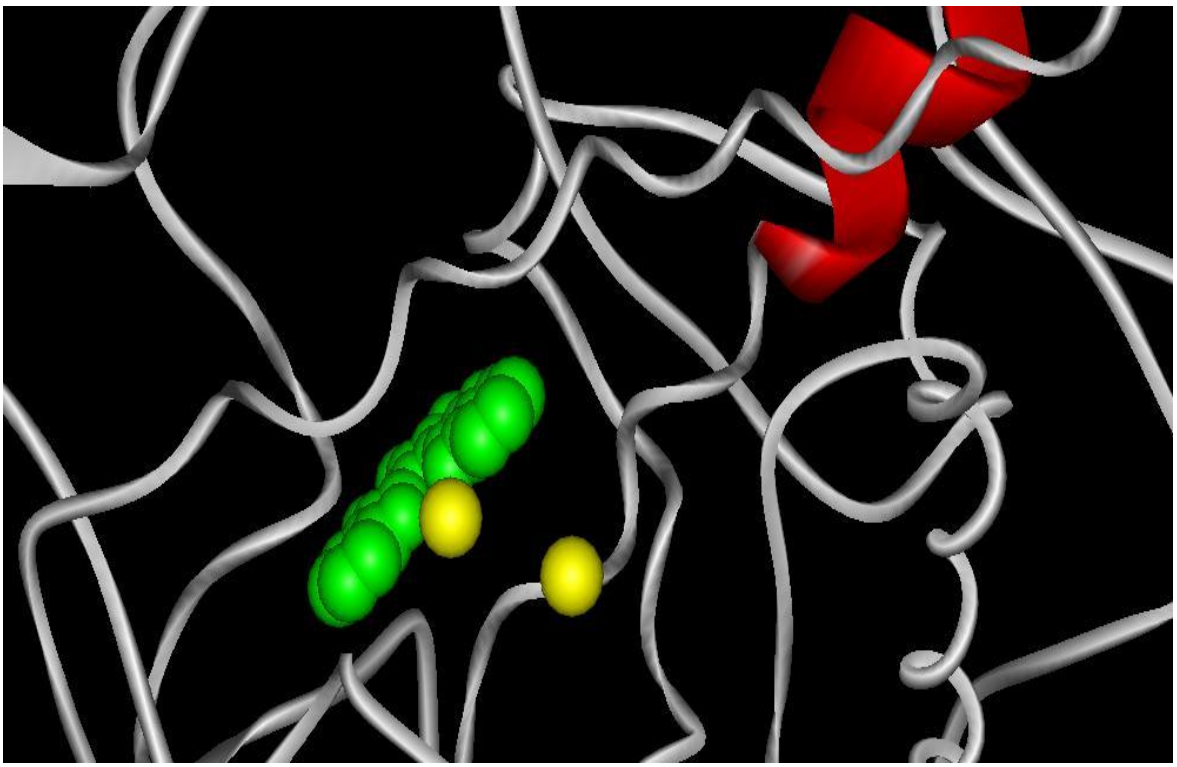

Fig.6. Computational docking results for tyrosinase and ligand. The green one is ligand and Yellow ones are Cu atoms of active site.

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