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Inhibition of Ascorbic Acid on Lotus Rhizome Polyphenol Oxidase: Inhibition Kinetics and Computational Simulation

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Abstract

Polyphenol oxidase(PPO) is widely known to be involved in enzymatic browning reaction in many fruits and vegetables including lotus rhizome with different catalytic mechanisms. In this study, the inhibitory effect and mechanisms of action of ascorbic acid (AA) on the lotus rhizome PPO were investigated using inhibition kinetics and computational simulation. The lotus rhizome PPO was extracted with PBS (pH 7.0), fractionated with ammonium sulphate, concentrated, and purified with DEAE-52(2.6×30 cm) and Sephadex G-75(2.6×60 cm) chromatography. The active fractions were pooled and the PPO activity was determined to be 2627.36Units/mg. AA exhibited inhibition on lotus rhizome PPO with residual activity of 13.79% at concentration of 0.08mM and IC_{50} of 0.045mM. Kinetic analyses determined by Lineweaver-Burk plots showed that ascorbic acid was reversible and competitive inhibitor to the enzyme. The 3D structure of the lotus rhizome PPO was simulated by SWISS-MODEL program and molecular docking was performed between PPO and its ligands (catehol and AA) by SYBYL-X 2.0. Simulation results showed that AA and catechol compete with the binding site of the PPO active center for its stronger affinity with the enzyme. In conclusion, the AA was established as a competitive inhibitor of lotus rhizome PPO, which provides a theoretical basis for it as an anti-browning agent in storage and preservation of lotus rhizome.

Keywords: Lotus rhizome, Polyphenol oxidase, Computational simulation, Inhibition mechanism

1. Introduction

Lotus rhizome (*Nelumbo nucifera* Gaertn.) is a perennial aquatic plant belongs to the family *Nelumbonaceae* and genus *Nelumbo*. It is famous for both economic and ornamental plant (Guo, 2009)**.** Moreover it is one of the most popular vegetables all over the world due to its crispness, attractive white color and abundant of nutrients (Xing *et al.,* 2010). However, lotus rhizome products such as fresh-cut slices are always deemed to be a lower quality for the faster rate of surface enzymatic browning during peeling and cutting (Xing *et al.,* 2010), which affects sensory evaluation of consumers.

Enzymatic browning commonly occurs in many fruits and vegetables after harvest during handling, processing and storage (Busch,1999; Jang *et al.,* 2002), considered an undesirable reaction due to discoloration, off-flavors, softening and loss of nutritional quality for the breakdown of vitamins (Martinez and Whitaker, 1995; Weemaes *et al.,* 1997). The discoloration of fruits and vegetables by enzymatic browning causes millions of dollars in losses per year to the food industry (Grotheer *et al.,* 2012). Enzymatic browning is mainly caused by polyphenol oxidase (PPO), which catalyses the oxidation of phenolic compounds to quinones in the presence of oxygen and then subjected to further reactions. Although these quinones enzymatically catalysed or not, but leading to the formation of pigments that ultimately decrease the value of the fruits and vegetables (Krapfenbauer *et al.,* 2006; Özoğlu & Bayındırlı, 2002).

Use of anti-browning agents is a common approach for preventing enzymatic browning (Arslan and Dogan, 2005). Sulfites, acidifiers, chelators, reducing agents, calcium ascorbate, and L-cysteine, have been commonly used for controlling PPO-related enzymatic browning in fruits and vegetables (Abbott *et al.,* 2004; Bhagwat, 2004; Fayad *et al.,* 1997; Karaibrahimoglu *et al.,* 2004). Among these, sulfites and ascorbic acid (AA) were most frequently used. The former have been restricted by the U.S. Food and Drug Administration for its adverse health effects (Coetzer et al., 2001). The ascorbic acid (AA) has been reported to control effectively enzymatic browning of fruits and vegetables (Gorny *et al.,* 2002; El-Shimi, 1993; Komthong *et al.,* 2007; Jang and Moon,

2011; Landi *et al.,* 2013)**.**

Studies have demonstrated the effect of AA on the inhibition of lotus rhizome browning (Jiang *et al.,* 2014; Xing *et al.,* 2012)**.** However, there is no clear picture of the mechanism of AA in regarding to inhibition of lotus rhizome browning. The aim of the present study is to investigate the effect of AA on lotus rhizome PPO activity and figure out the mechanism of PPO inhibition by AA using the method of kinetic analysis and computational simulation in order to better understand and control browning in fruits and vegetables.

2. Materials and methods

2.1Materials and chemicals

Lotus rhizome (*Nelumbo nucifera* Gaertn.) was purchased from the market of Huazhong Agricultural University at Wuhan, PR China, washed and removed the dust. Ascorbic acid (AA) and catechol were purchased from Sigma Chemical Co (St. Louis, USA). The other chemicals with analytical-grade were obtained from Beijing Chemical Reagent Company.

2.2 *Extraction and purification of PPO*

Lotus rhizome (200 g) were cut into small pieces and homogenised in 400 ml of 100 mM PBS (pH 7.0) containing 10g/L PVPP for 2 min. Then the homogenate was filtered through eight layers of gauze and centrifuged at 12500×g for 20 min. The supernatant was collected as crud enzyme. All extraction procedures were carried out at 4°C.

The crude enzyme was treated with 40 to 85% saturated solid ammonium sulfate. The precipitate was obtained by centrifugation at 12000×g for 20 min, dissolved in a small amount of 50mM PBS (pH 7.0) and then dialysed against 10mM PBS (pH 7.0) until no sulphate could be detected. The prepared solution was centrifuged at 12000×g for 20 min and supernatant was collected. The supernatant was chromatographed by a DEAE-52 column (2.6×30 cm) pre-equilibrated with 10mM PBS (pH 7.0). The column was eluted with a linear gradient (0-0.5M NaCl) of 10mM PBS (pH 7.0) at a flow rate of 30ml/h and 5mL per tube as a fraction. Fractions with enzyme activity were pooled, dialysed against 10mM PBS (pH 7.0) and freeze dried. The freeze-dried enzyme was redissolved in a small amount of 100mM PBS (pH 7.0) and further chromatographed on a Sephadex G-75 column (2.6×60 cm) eluted with 100 mM PBS (pH 7.0) at a flow rate of 12 ml/h and 6mL per tube as a fraction. All procedures were carried out at 4℃. Fractions with enzyme activity were pooled, collected as purified enzyme and freeze dried for subsequent analysis.

The protein was determined by the following the method of Lowry *et al.,* 1951 with bovine serum albumin (BSA) as the standard.

2.3 *Assay of PPO activity and kinetic study*

The lotus rhizome PPO activity assay was performed as reported by (Sukhonthara and Theerakulkait, 2012) with slight modifications. The increase in absorbance at 420nm with a spectrophotometer (Spectrum 754PC, Spectrum Instruments Co., Ltd, Shanghai, China) at 35°C was measured.

A 0.1mL of enzyme extract was mixed with 1.9mL of PBS 50mM (pH 7.0) and then the reaction was initiated by adding 1.0mL of 0.2M catechol substrate solution in 50mM PBS (pH 7.0). For inhibition determination, the 1.9mL of PBS was replaced by 0.9mL of 50mM PBS (pH 7.0) and 1.0mL of AA at various concentrations, and then the mixture was incubated with 0.1mL of enzyme extract for 10min followed by adding 1.0mL of substrate solution. As a control, the inhibitor was replaced by1.0mL of distilled water. A unit of enzyme activity was defined as the change of 0.001 in the absorbance value per minute under the conditions of the assay.

The inhibition type was assayed by the Lineweaver-Burk plot, and the inhibition constant was determined by the second plots of the apparent Km/Vm or 1/Vm versus the concentration of the inhibitor (Shi *et al.,* 2005). The measurements were performed in triplicate.

2.4 *Molecular modeling and computational docking study*

Since the crystallographic structure of lotus rhizome PPO has not been determined, the 3D structure of the enzyme is constructed by homology modeling. The complete amino acid sequence of the lotus rhizome PPO was

retrieved from NCBI protein sequence database (accession: ADP89908). The BLAST algorithm against Protein Data Bank (PDB) was used to search suitable template and as a result, the known crystal structure of Grenache (*Vitis vinifera)* PPO (PDB ID: 2P3X) (Viradore *et al.,* 2010), was selected as template in subsequent homology modeling for lotus rhizome PPO with the highest identity of 65.88%. The pair wise sequence alignment of the template sequence and the lotus rhizome PPO sequence was done using online Clustal W program with default parameters (Larkin *et al.,* 2007).

Homology modeling of the lotus rhizome PPO model was performed by three homology modeling program SWISS-MODE (Schwew, 2003). The stereochemical quality and energetic architecture of the model obtained by SWISS-MODEL was validated by inspection of the Phi/Psi Ramachandran plot obtained from PROCHECK (Love II *et al.,* 2003; Laskowski *et al.,* 1993).

After removing water molecules and adding all the hydrogen atoms, Gasteiger-Huckle charges were assigned to the predicted model. The energy minimization of predicted model was performed by Powell minimization to reach a root mean square deviation(RMSD) gradient energy of 0.05 kcal· mol^{-1}· A^{-1} using AMBER7 FF99 force field in SYBYL-X 2.0.

The ligand compounds were drawn by Sketch function and optimized in SYBYL-X 2.0 (Tripos Inc., St. Louis, MO). Relevant energy minimization of target compounds was conducted using Tripos Force Field (distance-dependent dielectric) with atom charge calculated by Gasteiger-Hückel method to reach a final energy convergence gradient value of 0.001 kcal/mol. The optimized structures were used as reasonable starting conformations for subsequent molecular docking.

Molecular docking has been performed between lotus rhizome PPO model and ligands including its substrates and inhibitors by using Surflex-Dock module in SYBYL-X 2.0 with default setting (Kellenberger *et al.,* 2004; Jian, 1996). The resultant docking scores were expressed in -log(Kd) unit, in which Kd is the dissociation constant (Jian, 2007) and converted to the free energy of binding (kcal/mol), which was calculated by following the equation given below:

Free energy of binding = RT $\log_e$ (10^{-pKd}), where RT=0.59 kcal/mol (Holt *et al.,* 2008).

2.5 *Statistical analysis*

All experiments were repeated three times with duplicate samples in each repetition. The dose-response analysis of AA on the enzymatic activity was performed using linear regression in Origin8.0. Data were analysed for statistical significance using the Proc Mixed procedure of SAS (SAS Institute, Cary, NC).

3. Results and Discussion

3.1*Purification of PPO*

In order to get purified enzyme, the PPO from lotus rhizome was extracted by PBS (pH 7.0) and fractionated with ammonium sulphate, and then the preparation was chromatographed by DEAE-52 column and then Sephadex G-75 column. The purification chromatography was showed in Fig.1. As a result of DEAE-52 column (Fig.1a), three elution peaks were detected at 280nm and the second one was assayed as PPO active fractions, which were pooled and collected for subsequent purification with specific activity 857.72 Units/mg. As a result by Sephadex G-75 column (Fig.1b), one single elution peak was detected. The active fractions were pooled and the specific activity was 2627.36Units/mg with 99.71-fold purification compared to the crude extract. The purified enzyme was used in subsequent inhibition kinetic study.

3.2 Effect of AA on PPO activity in vitro

Lotus rhizome PPO activity changes were assayed in the presence of AA at different concentrations (0, 0.02, 0.04, 0.06, 0.08mM). As shown in the Fig.2, the PPO activity was inactivated by AA in a dose-dependent manner and the inhibitory concentration leading to 50% enzyme activity lost (IC_{50}) was estimated to be 0.045mM. At 0.08mM AA, only 13.79% of the PPO activity was determined.

The inhibition mechanisms on lotus rhizome PPO by AA for the oxidation of catechol were studied. As showed in Fig.3, the relationship of enzyme activity to the enzyme concentration in the presence of different concentrations of AA, the plots of the remaining enzyme activity versus the concentrations of enzyme in the presence of different concentrations of AA gave a family of straight lines, which all passed through the origin.

Increasing of AA concentration resulted in descent of the slope of the line, indicating that the inhibitor on the enzyme was a reversible course. The presence of AA did not bring down the amount of the efficient enzyme, but just resulted in the inhibition and the decrease of the catechol oxidation activity of the enzyme.

3.3 Inhibition kinetics of AA on PPO

The inhibition kinetics of AA on lotus rhizome PPO were analysed by Lineweaver-Burk plots as shown in Fig.4. The plots of 1/*v* versus 1/[S] gave a family of straight lines with different slopes but they were intersected in one another in the *Y*-axis. The values of V_{max} remained the same but the values of K_m increased with increasing concentrations of the inhibitor, which indicates that AA is a competitive inhibitor for lotus root PPO. The result demonstrated that AA could only bind with free enzyme. According to the secondary plot (did not show), the inhibition constant (K_I) for AA binding with the enzyme was 4.98mM.

3.4 Molecular docking of AA to PPO

Because the crystallographic structure of lotus rhizome PPO is not available, the known crystal structure of Grenache *(vitis vinifera*) PPO (PDB ID: 2P3X) based on the result of the BLAST algorithm against Protein Data Bank (PDB) with the highest identity of 65.88% was selected as the template to simulate the 3D structure of the lotus rhizome PPO.

The interactions of catechol and AA with the active site of lotus rhizome PPO were studied with the use of Surflex-Dock module in SYBYL-X 2.0 and a binding pocket is indicated in box. The results of docking the ligands with the enzyme are shown in Fig.5. As it is clear, the catechol is embedded in the spiral of the PPO structure. Two hydrogen bonds are formed between catechol hydrogen atom and the oxygen atoms of GLU280 and ASN281. The free energy of binding is -4.58kcal/mol. This chemical interaction make PPO catalyze the oxidation of catechol to quinone in the presence of oxygen. Interestingly, when the AA was docked with PPO, six hydrogen bonds are formed between AA and amino acid residues in the active site, with the free energy of binding of -4.63kcal/mol. Three are formed between AA hydrogen atoms and the oxygen atoms of GLU280 and ASN281, two are formed between AA oxygen atom and the hydrogen atoms of ASN281, and one is formed between AA oxygen atom and the hydrogen atom of HIS284. Therefore, it could be inferred that AA inhibits the activity of PPO by preventing the substrate (catechol) from binding with the key amino acid residues (GLU280 and ASN281) in the active site in the competitive process for its stronger affinity with the enzyme, which is consistent with the result of inhibition kinetics.

4. Conclusion

The shelf-life of lotus rhizome products is usually limited by rapid browning caused by polyphenol oxidase (PPO). In this study, the inhibitory effect and associated mechanisms of AA on the lotus rhizome PPO were investigated. The principal findings are concluded as follows: PPO from lotus rhizome was purified by Chromatography. AA inhibited lotus rhizome PPO in a dose-dependent manner with a low IC_{50}. The inhibition kinetics and binding modes of substrate catechol and inhibitor AA with lotus rhizome PPO were determined using Lineweaver-Burk plots and Surflex-dock and showed that AA inhibited the lotus rhizome PPO activity through a competitive inhibition mechanism by competing with substrate in the enzyme active site pocket.

The study focused on the inhibition of AA on lotus rhizome PPO using catechol as substrate in the specific assay condition. In order to illustrate the inhibition mechanisms of AA comprehensively, more research should be done to investigate the inhibition effect of AA on the enzyme from different plant tissue for the catalysis of other substrates such as L-DOPA and L-tyrosine under the different assay conditions.

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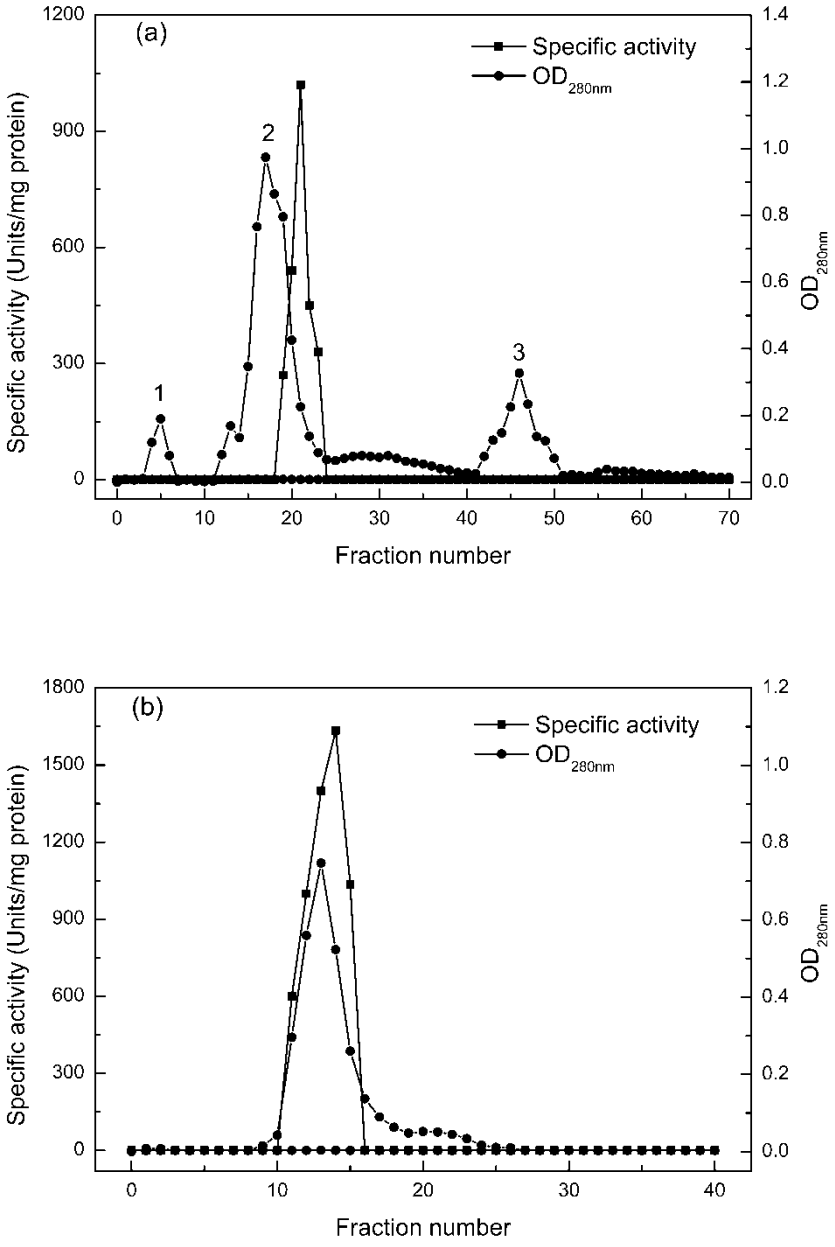


Fig.1. Column chromatography of lotus rhizome PPO on DEAE-52(a) Sephadex G-75 (b). 1, 2 and 3 represent the number of elution peak monitored at 280nm.

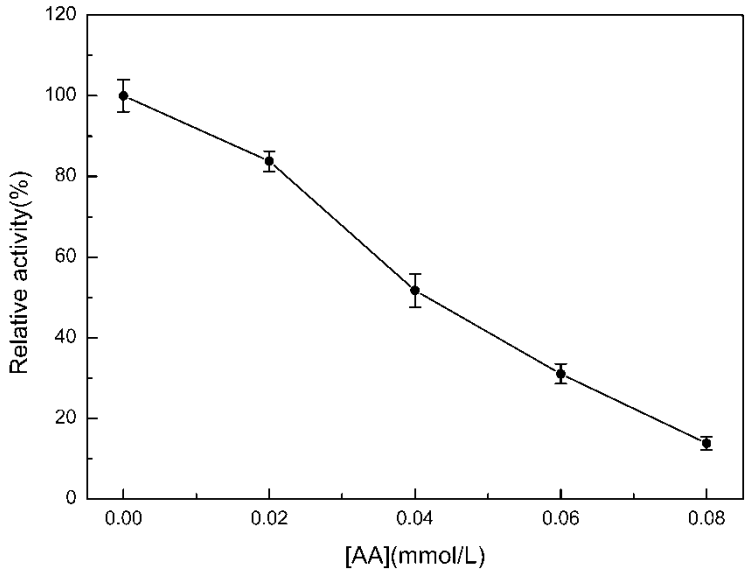


Fig.2. Inhibitory effect of AA on lotus rhizome PPO relative activity for the oxidation of catechol

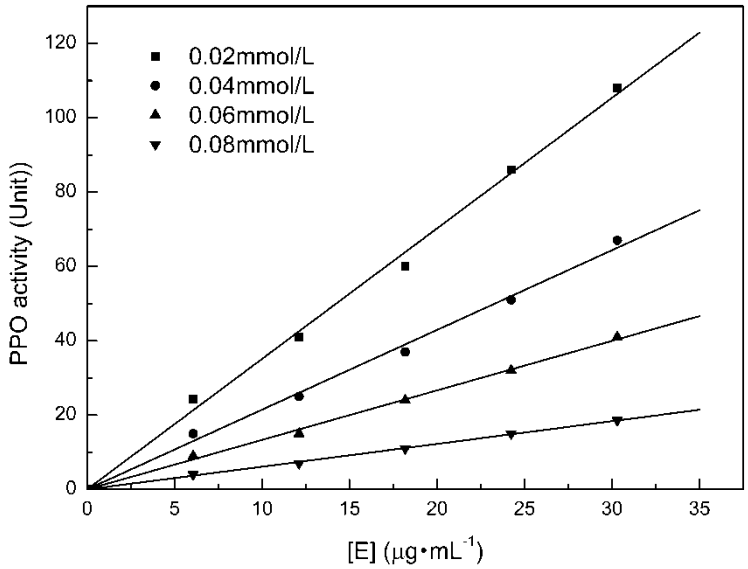


Fig.3. Effect of concentration of lotus rhizome PPO on its activity for the oxidation of catechol at different concentrations of AA.

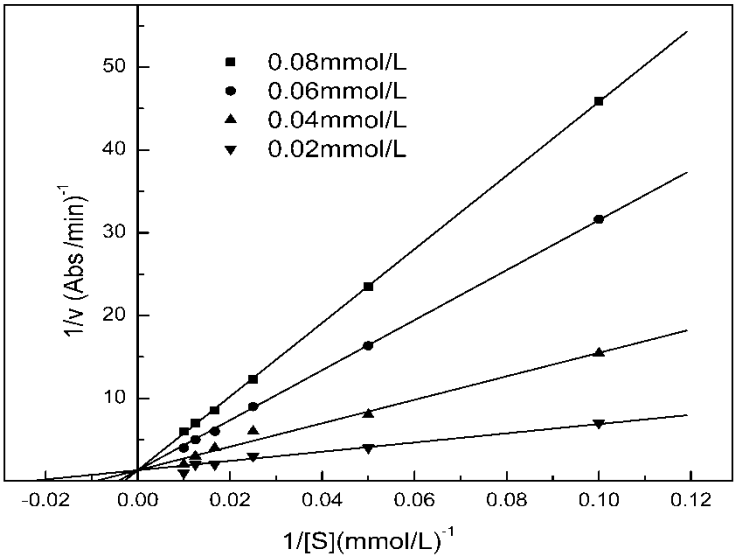
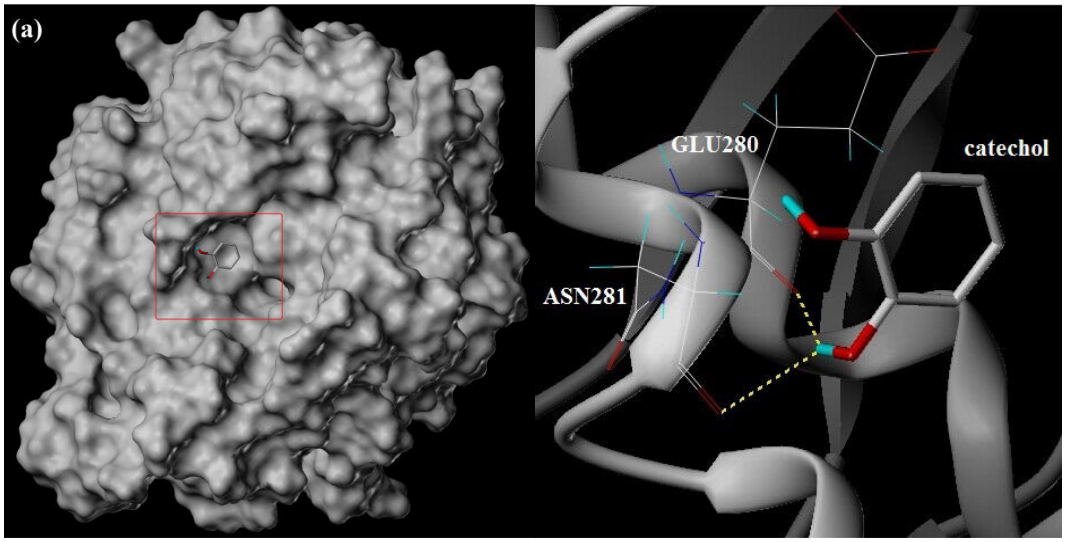


Fig.4. Lineweaver-Burk plots for inhibition of AA on lotus rhizome PPO for the catalysis of catechol.



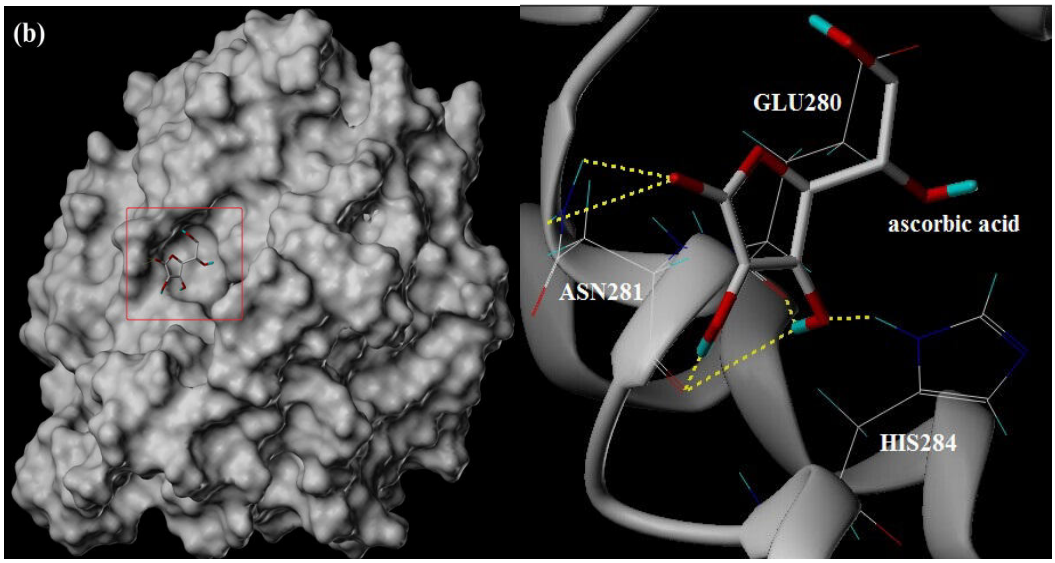


Fig.5. Key interactions of catechol (a) and AA (b) with the active site of lotus rhizome PPO by computational simulations (Surflex-Dock) in SYBYL-X 2.0. Binding of ligands in the PPO active site is indicated. Ligands are in capped sticks model and the histidine, asparagine, glutamic acid residues are in line models. Yellow dashed lines represent hydrogen bonds.