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Inhibitory effects of 2-hydroxybenzaldehyde on the activity of phenoloxidase from *Pieris rapae* (Lepidoptera) larvae

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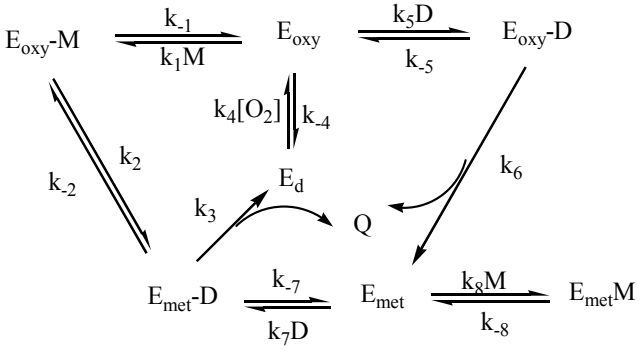
Phenoloxidase (PO) is a key enzyme in insect development, responsible for catalyzing the hydroxylation of tyrosine into *o*-diphenols and oxidation of *o*-diphenols into *o*-quinones. In the present study, the kinetic assay for PO from *Pieris rapae* (L.) larvae was performed in air-saturated solutions and its kinetic behavior in the oxidation of L-tyrosine (a monophenol) and L-DOPA (L-3, 4-dihydroxyphenylalanine, a diphenol) was studied. The inhibitory effects of 2-hydroxybenzaldehyde (2-HBD) on the monophenolase and diphenolase activities of PO were also studied. Results showed that 2-HBD inhibit both the monophenolase and diphenolase activities of PO. The lag period of L-tyrosine oxidation catalyzed by the enzyme was lengthened and the steady-state activity of the enzyme sharply decreased. The inhibitor was found to be noncompetitively reversible with a K_I ($K_I = K_{IS}$) of 1.21 mmol/L and an estimated IC_{50} of 8.08 ± 0.11 mmol/L for monophenolase and 4.14 ± 0.08 mmol/L for diphenolase activities. In the time-course of oxidation of L-DOPA catalyzed by the enzyme in the presence of different concentrations of 2-HBD, the rate decreased with increasing time until a straight line was reached. The microscopic rate constants for the reaction of 2-HBD with the enzyme were determined.

Keywords: Phenoloxidase, Inhibition kinetics, 2-Hydroxybenzaldehyde, Monophenolase, Diphenolase, *Pieris rapae* (L.)

Phenoloxidase (PO) (EC 1.14.18.1), a copper-containing bifunctional enzyme, also known as tyrosinase is common in microorganisms, animals and plants[1,2]. In insects, PO is uniquely associated with three physiologically important biochemical processes: sclerotization of insect cuticle; wound healing and defensive encapsulation; and melanization of foreign organisms[4]. It catalyzes two distinct reactions of melanin synthesis — hydroxylation of monophenol (monophenolase activity) and conversion of *o*-diphenol into the corresponding *o*-quinone (diphenolase activity)[3]. The production of *o*-quinones by PO is the initial step in the biochemical cascade of sclerotization, quinone tanning and melanin biosynthesis, the processes that play several major roles in the insect development and immunity.

PO exists in three states — E_{met}, E_{deoxy} and E_{oxy}[5-7] and structural models for the active site of these forms have been proposed[8-11]. The reactions catalyzed by these three forms of the enzymes are summarized in Scheme 1. Here M and D represent two different substrates monophenol (L-tyrosine) and diphenol



Scheme 1

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Abbreviations: PO, phenoloxidase; 2-HBD, 2-hydroxybenzaldehyde; M, monophenol; D, *O* -diphenol; Q, quinone; E_{oxy}, *oxy*-PO (with Cu^{2+}-O_2^{2-}-Cu^{2+} in the active site); E_{met}, *met*-PO (with Cu^{2+}-Cu^{2+} in the active site); E_d, *deoxy*-PO (reduced form of PO with Cu^{+}-Cu^{+} in the active site); v_0, initial rate; $[E]_0$, initial PO concentration; IC_{50}^{D}, concentration to inhibit 50% of PO activity towards D; IC_{50}^{M}, concentration to inhibit 50% of PO activity towards M; K_m^D, apparent Michaelis constant of PO towards D; V_m^D, apparent maximum steady-state rate of PO towards D.

(L-DOPA), Q, the product quinone, E_{oxy} (*oxy*-PO), E_{met} (*met*-PO) and E_d (*deoxy*-PO), the three forms of the enzyme, E_{oxy}-M, E_{met}-M, E_{oxy}-D, E_{met}-D, the enzyme-substrate complexes, and k and k_- the rate constants for the forward and reverse reactions of each step. The *oxy*-PO starts the turnover by acting on M, which is hydroxylated to *met*-PO-diphenol intermediate (E_{met}-D). At this point, the enzyme can oxidize D to *o*-quinone (Q) to produce *deoxy*-PO (E_d) or release D to make the met (E_{met}) form, which binds with M to produce the inactive form E_{met}-M. If Q is quite unstable, as occurs in the case of *o*-dopaquinone, D is recycled to the medium through intramolecular cyclization and further redox steps involving transformation of E_{met} form of the enzyme (which is inactive towards M) into active E_{oxy} form (which is active towards M), giving rise to a characteristic lag period[12-14]. In such cases, the system would reach a steady state.

Recently, the crystal structures of copper-bound and metal-free tyrosinase have been determined in a complex with ORF378 designated as a "caddie" protein, because it assists with transportation of two Cu(II) ions into the tyrosinase catalytic center. These structures suggest that the caddie protein covers the hydrophobic molecular surface of tyrosinase and interferes with the binding of a substrate tyrosine to the catalytic site of tyrosinase. The crystallographic study also shows that the tyrosinase active center formed by dinuclear coppers is flexible during catalysis[11].

Two copper atoms in the active site of PO have become targets of inhibition studies, and PO inhibitors have increasingly been used in medicinal and cosmetic products[15,16]. Flavor compounds from olive oil and flavonols from saffron flower apart from copper chelators are known to inhibit mushroom tyrosinase[17,18]. Since PO is one of the key enzymes in insect molting process[19], screening of its inhibitors might provide clues for the control of insect development[20-22]. In the present study, inhibitory effect of 2-hydroxybenzaldehyde (2-HBD) on PO monophenolase and diphenolase activities in *Pieris rapae* larvae has been investigated and the inhibition kinetics of 2-HBD is discussed in detail.

Materials and Methods

Insects and reagents

Pieris rapae (L.) larvae were reared on Chinese cabbage *Brassica parachinesi* (Bailey) in a greenhouse at 25 ± 1°C with a 14:10 h light:dark photoperiod. The 5th instar larvae were gathered for the experiments. L-DOPA (L-3, 4-dihydroxyphenyl-alanine), L-tyrosine and dimethyl sulfoxide (DMSO) purchased from Aldrich Chemical Co. (Milwaukee, WI, USA), 2-hydroxybenzaldehyde (2-HBD) from Sigma Chemical Co. (St. Louis, MO, USA) and Sephadex G-100 from Amersham Pharmacia Biotech (Uppsala, Sweden) were used. All other reagents were local products of analytical grade. The water used was re-distilled and ion-free.

Enzyme purification

The 5th instar larvae were homogenized in a 5-fold weight of ice-cold 0.2 mol/L sodium phosphate buffer (*p*H 6.9). The homogenate was centrifuged for 30 min at 9310 × *g* after extraction for 2 h. The supernatant under the fat layer was collected as the crude enzyme extract and was brought to 35% saturation with solid ammonium sulfate. The precipitate was again centrifuged at 9310 × *g* for 30 min, and then re-dissolved in a minimum volume of the same phosphate buffer and dialyzed against the 0.01 mol/L sodium phosphate buffer (*p*H 6.9). The dialyzed solution was loaded on to a Sephadex G-100 column (2.5 × 60 cm), equilibrated with 0.01 mol/L sodium phosphate buffer (*p*H 6.9) and the series fractions having high activities were collected (3 ml per tube). The enzyme activity of every tube was assayed and the gel filtration pattern was portrayed. The second process increased the specific activity up to 6.22-fold of that of crude enzyme extract. All procedures were carried out at 4°C.

Enzyme assays

All assays were carried out in air-saturated solutions. Enzymatic activity was assayed by monitoring dopachrome formation at 475 nm [$\varepsilon = 3700\ mol^{-1}\cdot L\cdot cm^{-1}$][23] accompanying the oxidation of the substrate (L-tyrosine or L-DOPA). The assay was performed as previously described[24] with slight modifications. The reaction media (2 ml) for PO activity contained 1 mmol/L L-DOPA or 4 mmol/L L-tyrosine in 0.1 mol/L Na_2HPO_4-NaH_2PO_4 buffer (*p*H 6.9), the indicated concentration of 2-HBD and 3.3% DMSO. Under the method, different amounts of 2-HBD were dissolved in DMSO solution and added to the test tube. To this mixture was added 1.9 ml of substrate solution in Na_2HPO_4-NaH_2PO_4 buffer (pre-warmed to 37°C) and 0.1 ml of aqueous enzyme solution. The final solution was immediately

monitored for 100 s for formation of dopachrome by measuring the linear increase in optical density at 475 nm at a constant temperature of 37°C. Absorption was recorded using a Hitachi UV-2201 spectrophotometer. The inhibition extent was expressed as the concentration to inhibit 50% of PO activity (IC_{50}). The kinetic and inhibition constants were obtained by the previously described method[14,24].

Results

Kinetics of PO for oxidation of L-tyrosine and L-DOPA

The kinetic behavior of PO was studied during oxidation of different concentrations of L-DOPA and L-tyrosine and progress curves were determined for the enzyme at each concentration. When the monophenolase activity of PO was assayed using L-tyrosine as the substrate, a remarkable lag period, characteristic of monophenolase activity was observed with the simultaneous appearance of the first stable product dopachrome (Fig. 1, curves 1-4). The system reached a constant rate after the lag period, which was estimated by extrapolation of linear portion of the product accumulation curve to the abscissa[25]. After the reaction system reached steady state, the curve of product increased linearly with increasing reaction time. When the diphenolase activity of PO was assayed using L-DOPA as the substrate, the reaction immediately reached a steady-state rate (Fig. 2A, curve 0). It is shown as a straight line that passes through the original point of the coordinate with a slope equal to the initial rate (v_0). The relationship of initial rate and concentration of substrate (L-DOPA) followed Michaelis-Menten kinetics. Kinetic parameters for the diphenolase activity of PO are shown in Fig. 3 (line 0) as a Lineweaver-Burk plot. The apparent Michaelis constant (K_m^D) was 1.26 ± 0.04 mmol/L and the apparent maximum velocity (V_m^D) was 0.50 ± 0.05 mmol/L/min.

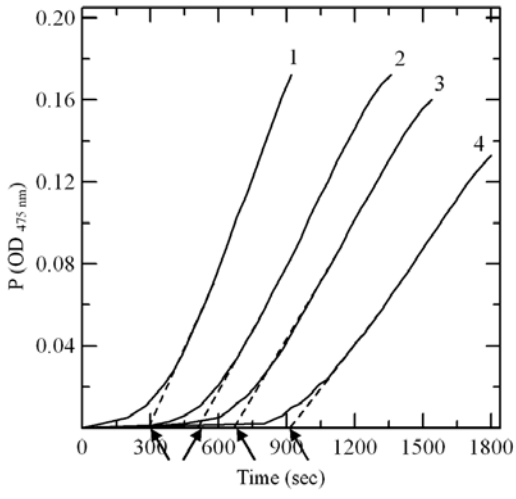


Fig. 1—Progress curves for inhibition of PO from *P. rapae* (Lepidoptera) larvae by 2-HBD. Curves 1, 2, 3 and 4 show the effects of 2-HBD on the monophenolase activity of PO [The reaction media contained L-tyrosine 4 mmol/L in 0.1 mol/L Na_2HPO_4-NaH_2PO_4 buffer *p*H 6.9. 2-HBD concentrations were (1) 0, (2) 2.0 mmol/L, (3) 4.0 mmol/L, (4) 6.0 mmol/L. The arrows show the lag period]

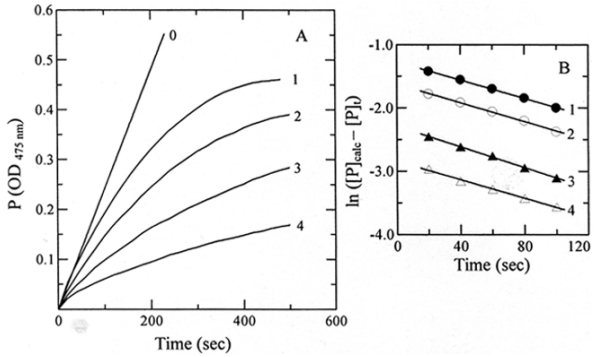


Fig. 2—Course of substrate reaction in presence of different concentrations of 2-HBD [The final assay conditions: 2 ml system containing 0.1 mol/L Na_2HPO_4-NaH_2PO_4 buffer *p*H 6.9, 1 mmol/L L-DOPA as substrate and different concentrations of 2-HBD. (A) Time-course of substrate oxidation reaction. The concentrations of 2-HBD for curves 0-4 were 0, 2.0, 4.0, 6.0 and 8.0 mmol/L, respectively; (B) Plots of $\ln([P]_{calc}-[P]_t)$ *vs.* time. Data were taken from curves 1-4 in (A)]

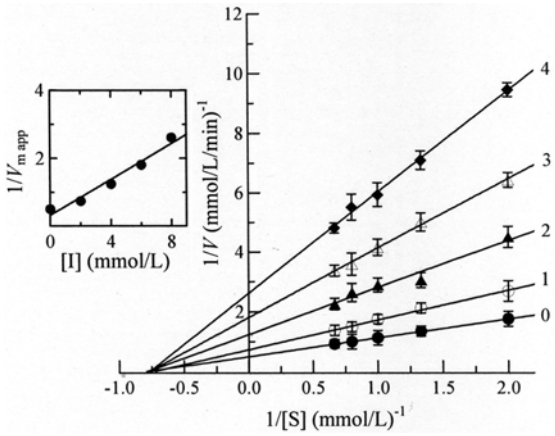


Fig. 3—Lineweaver-Burk plots for inhibition of 2-HBD on diphenolase activity of PO for catalysis of L-DOPA at 37°C, *p*H 6.9 [Assay conditions: 2 ml system containing 0.1 mol/L Na_2HPO_4-NaH_2PO_4 buffer *p*H 6.9, different concentrations of L-DOPA as substrate and different concentrations of 2-HBD. Concentrations of 2-HBD for curves 0-4 were 0, 2.0, 4.0, 6.0 and 8.0 mmol/L, respectively. The inset represents the plot of $1/V_{m\ app}$ *vs.* 2-HBD concentration for determining the inhibition constants K_I]

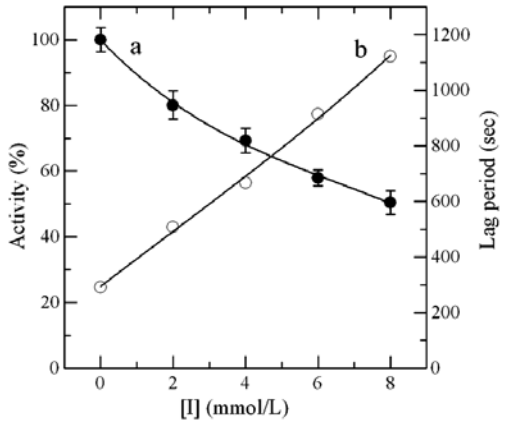


Fig. 4—Effect of 2-HBD on the steady-state rate of monophenolase activity (a) and the lag period of PO (b) for oxidation of L-tyrosine [Assay conditions: as described for Fig. 1, except using 4 mmol/L of L-tyrosine as substrate]

Table 1—Kinetics parameters and microscopic inhibition rate constants of PO from *Pieris rapae* (Lepidoptera) larvae by 2-HBD

IC_{50}^{D}	4.14 ± 0.08 mmol/L
IC_{50}^{M}	8.08 ± 0.11 mmol/L
K_m^D	1.26 ± 0.04 mmol/L
V_m^D	0.50 ± 0.05 mmol/L·min
Inhibition	Reversible
Inhibition type	Non-competitive
K_I	1.21 mmol/L
K_{IS}	1.21 mmol/L
k_{+0}	3.28×10^{-3} mmol/L·sec
k_{-0}	8.16×10^{-6} sec^{-1}

Effect of 2-HBD on monophenolase activity of PO

The effect of 2-HBD on monophenolase activity of PO was tested using L-tyrosine as the substrate. Fig. 4 shows that 2-HBD inhibited monophenolase activity at the steady-state rate (curve a) and lengthened the lag period of PO (curve b) proportional to its concentration. The steady-state rates, which were the slopes of linear portions of the kinetic curves decreased accordingly. Eight mmol/L of 2-HBD resulted in an extension of the lag period from 290 s to 1121 s. The IC_{50} value was estimated as 8.08 ± 0.11 mmol/L.

Effect of 2-HBD on diphenolase activity of PO

The effect of 2-HBD on diphenolase activity of PO was examined by measuring oxidation of L-DOPA. The results showing kinetics of product generation at different concentrations of inhibitor are represented as straight lines that pass through the original points. The slopes of the lines decreased with increasing 2-HBD concentrations (Fig. 2A, lines 1-4), indicating a positive correlation between the inhibition of diphenolase activity and the concentration of 2-HBD, as shown in Fig. 5. The IC_{50} value was estimated as 4.14±0.08 mmol/L and is summarized in Table 1.

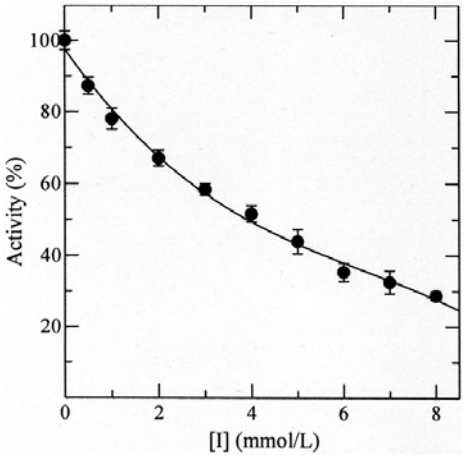


Fig. 5—Effect of 2-HBD on the PO diphenolase activity from *P. rapae* larvae for the catalysis of L-DOPA at 37°C [Assay conditions: 2 ml 0.1 mol/L Na_2HPO_4-NaH_2PO_4 buffer *p*H 6.9, containing 1 mmol/L L-DOPA]

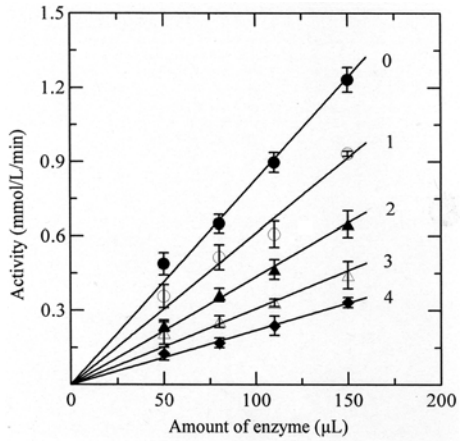


Fig. 6—Effects of different concentrations of 2-HBD on the activity of PO from *P. rapae* (Lepidoptera) larvae for the catalysis of L-DOPA at 37°C [Assay conditions were as described for Fig. 4, except for change the PO concentrations. Concentrations of 2-HBD for curves 0-4 were 0, 2.0, 4.0, 6.0 and 8.0 mmol/L, respectively]

Inhibition of PO diphenolase activity by 2-HBD is reversible and non-competitive

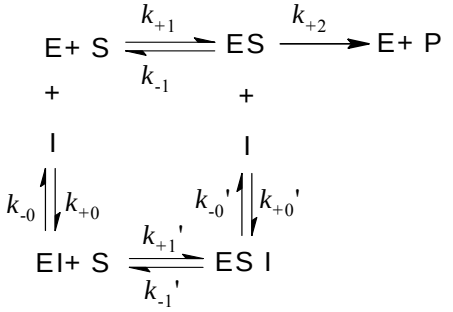
The relationship between diphenolase activity and enzyme concentration in the presence of different concentrations of inhibitor was studied (Fig. 6). The plots of enzyme activity *vs*. enzyme concentration at different inhibitor concentrations yielded straight lines

passing through the origin. Higher inhibitor concentrations were associated with decreasing slopes, suggesting that inhibition of PO by 2-HBD was reversible. The presence of 2-HBD did not decrease the amount of enzyme needed for an efficient reaction; however, there was decrease in the enzyme activity.

The kinetic behavior of PO during oxidation of L-DOPA was also determined. Under the conditions employed in investigation, oxidation of L-DOPA by PO followed Michaelis-Menten kinetics. Fig. 3 shows that 2-HBD is a non-competitive inhibitor, since its increased concentrations results in a group of lines with a common intercept on the 1/[S] axis, but with different slopes. The apparent value of V_m decreased with no effect on K_m. The observed behavior showed that the inhibitor could combine with both the free enzyme (E) and enzyme-substrate (ES) complex and that the equilibrium constants for inhibitor binding with free enzyme K_I and enzyme-substrate complex K_{IS} were the same. The inhibition constant ($K_I = K_{IS}$) as obtained from a plot of vertical intercept ($1/V_{m\ app}$) *vs*. inhibitor concentration was 1.21 mmol/L (Fig. 3, inset).

Kinetics of substrate reaction in different concentrations of 2-HBD

The time-course of oxidation of L-DOPA catalyzed by PO in the presence of different 2-HBD concentrations is shown in Fig. 2A. At each concentration of 2-HBD, the rate decreased with increasing time until a straight line was approached, the slope of straight line was decreased with increasing 2-HBD concentrations. The above results, as analyzed by the Tsou method[26] suggested that formation of the inactive EI complex was a slow and reversible reaction. This reaction can be written as follows:



where E, S, P, and I denote enzyme, substrate, product and inhibitor (2-HBD), respectively; EI and ES are the enzyme-substrate and enzyme-inhibitor complexes; k_{+0} and k_{-0} are rates constants for the forward and reverse inhibition reactions of free enzyme; and k_{+0}' and k_{-0}' are rate constants for the forward and reverse inhibition reactions of ES. Usually, $[S]>>[E]_0$ and $[I]>>[E]_0$. Therefore, product formation can be expressed as follows:

$$[P]_t = \frac{v \cdot k_{-0}}{A} \cdot t + \frac{v \cdot (A - k_{-0})}{A^2} \cdot (1 - e^{-At}) \quad \ldots (1)$$

and

$$A = \frac{k_{+0} \cdot K_m + k_{+0}' \cdot [S]}{K_m + [S]} + k_{-0} \quad \ldots (2)$$

where $[P]_t$ is the concentration of product formed at time t, which is the reaction time; A is the apparent rate constant of inhibition; $[S]$ and $[I]$ are the concentrations of the substrate and inhibitor; and v is the initial rate of the reaction in the absence of the inhibitor, where $v = \frac{V_m \cdot [S]}{K_m + [S]}$. When t is sufficiently large, the curves become straight lines and the product concentration is written as $[P]_{calc}$:

$$[P]_{calc} = \frac{v \cdot k_{-0}}{A} \cdot t + \frac{v \cdot (A - k_{-0})}{A^2} \quad \ldots (3)$$

Combining Eqs 1 and 3 gives

$$[P]_{calc} - [P]_t = \frac{v}{A^2}(A - k_{-0}) \cdot e^{-At} \quad \ldots (4)$$

$$\ln([P]_{calc} - [P]_t) = -A \cdot t + \ln[\frac{v \cdot (A - k_{-0})}{A^2}] \quad \ldots (5)$$

where $[P]_{calc}$ is the product concentration expected from the straight-line portions of the curves as calculated from Eq. 3 and $[P]_t$ is the product concentration actually observed at time 't'. Plots of $\ln([P]_{calc} - [P]_t)$ *vs*. reaction time (t) give a series of straight lines at different concentrations of inhibitor ($[I]$) with slopes of -A. The apparent forward rate constant 'A' can be obtained from such graphs. From Eq. 3, a plot of $[P]_{calc}$ *vs*. time t gives a straight line with a slope of $\frac{v \cdot k_{-0}}{A}$. From the slope of the straight line, k_{-0} can be obtained.

Combining Eq. 2 and the Michaelis-Menten equation gives

$$\frac{A}{v} = \frac{K_m \cdot (K_{+0} + k_{-0})}{V_m} \cdot \frac{1}{[S]} + \frac{k_{+0}' + k_{-0}}{V_m} \quad \ldots (6)$$

A plot of A/*v* *vs.* 1/[*S*] gives a straight line with $K_m \cdot (k_{+0} + k_{-0})/V_m$ and $(k_{+0}' + k_{-0})/V_m$ as the slope and intercept of the straight line, respectively. K_m and V_m are known quantities from measurements of the substrate reaction in the absence of the modifier at different substrate concentrations and the rate constants k_{+0} can be obtained from the slope of the straight line, as summarized in Table 1. Plots of $\ln([P]_{calc} - [P]_t)$ *vs.* 't' produce a group of straight lines at different concentrations of 2-HBD with slopes of –A (Fig. 2B).

Kinetics of diphenolase reaction at different substrate concentrations in presence of 2-HBD

Fig. 7A shows the kinetic course of diphenolase reaction at different L-DOPA concentrations in the presence of 2-HBD (4 mmol/L). It was observed that when 't' was sufficiently large, both initial rate and slope of the asymptote increased with increasing substrate concentration. Similarly, plots of $\ln([P]_{calc} - [P]_t)$ vs. 't' give a group of straight lines at different concentrations of the substrate with slopes of '-A' as shown in Fig. 7B. The apparent forward rate constants can be obtained through suitable plots. Fig. 7C shows that the plot of A/*v* *vs.* 1/[*S*] gave a straight line. According to Eq. 6, slope of the straight line provides the value of $K_m \cdot (k_{+0} + k_{-0})/V_m$. The microscopic rate constant k_{+0} was then obtained from the slope.

Discussion

PO exhibits both monophenolase and diphenolase activities. During catalysis, it exists in three forms: E_{met}, E_{deoxy} and E_{oxy}. E_{oxy} has monophenolase activity, both E_{met} and E_{deoxy} have diphenolase activity and E_{deoxy} can combine with oxygen[27]. The E_{met} form does not act on monophenols, but has an affinity for them, binding them to form a dead-end complex that may explain the lag period prior to attainment of the steady-state rate[1,28]. Here, L-tyrosine and L-DOPA were used as substrates for monophenolase and diphenolase activity assays carried out in air-saturated solutions. The lag period could be estimated by extrapolation of the linear portion of the product accumulation curve to the X-axis. As shown in Fig. 1, the data indicate that the addition of 2-HBD lengthens the lag period of monophenolase formation, decreases the steady-state rates of both monophenolase and diphenolase activities and slows the rate of dopachrome formation.

The lag period is shortened or even abolished by the presence of reducing agents known as cofactors, especially *o*-diphenols such as quercetin[17,27]. Here, we

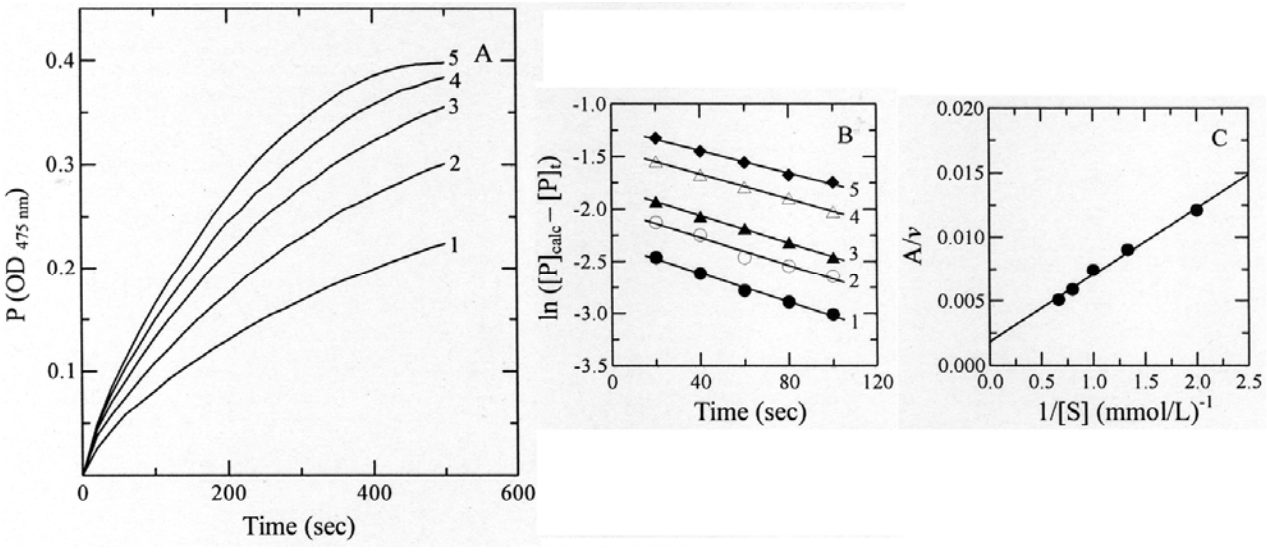


Fig. 7—Course of substrate reaction at different substrate concentrations in presence of 2-HBD [Experimental conditions were the same as those for Fig. 2, except the L-DOPA concentrations were different. (A) Time-course of substrate oxidation reaction. The concentrations of L-DOPA for curves 1-5 were 0.50, 0.75, 1.00, 1.25 and 1.50 mmol/L, respectively; (B) Plots of $\ln([P]_{calc} - [P]_t)$ *vs.* time. Data were taken from curves 1-5 in (A). (C) Plot of *A*/*v* vs. 1/[S]. The *A* were obtained from the slopes of the straight lines in (B)]

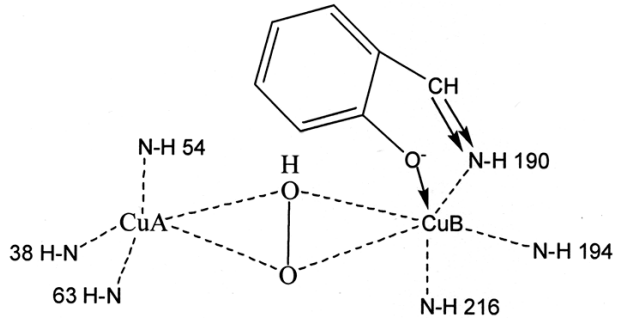


Fig. 8—Inhibitory mechanism model of 2-HBD on PO active center

found that 2-HBD could inhibit monophenolase activity and extend the lag period. It is likely that the inhibitor combines with the E_{met} and E_{oxy} forms to generate $E_{met}I$ and $E_{oxy}I$ complexes. The inhibition of PO by 2-HBD is reversible and non-competitive, indicating that the inhibitor can bind the free enzyme (E) to form both the EI and ESI complexes.

PO comprises several subunits, each having two binuclear copper sites. Each copper site combines with two smooth and one straight histidine ligand, and one O-O bond unites the two Cu^{+2} ions at each active site[11,29]. Several benzaldehyde derivatives[17] inhibit the oxidation of L-DOPA by phenoloxidase obtained from mushroom, presumably by forming a Schiff base between the aldehyde group of the inhibitors and a primary amino group of the enzyme. The aldehyde group generally reacts with biologically important nucleophilic groups such as sulfhydryl, amino, or hydroxyl groups. Formation of a Schiff base with a primary amino group of the enzyme is highly probable, because aromatic nucleus stabilizes it by conjugation[30].

As we expected, the inhibitor 2-HBD may be accommodated into the active pocket of PO. Thus, the covalent bond between the carbon atom in aldehyde group and nitrogen atom of primary amino group are formed. Also, the coordination bonds between the oxygen atom and Cu^{2+} of Cu^{2+}-O_2^{2-}-Cu^{2+} are also formed in the active site of PO, where the arrows located in Fig. 8. Besides, some hydrogen bonds are formed between 2-HBD reacting with amino group in the PO active site. Thus, the enzyme loses its catalysis activity for the above reasons; this appears the possible inhibitory mechanism of 2-HBD on PO.

As the PO plays a key role in normal insect development, it is possible to control pests by inhibiting the enzyme. This may form the basis for development of novel insecticides to replace traditional chemicals, many of which currently pose serious threat to the environment and/or have been associated with resistance, toxic residues and/or resurgence in target pest populations. In conclusion, this study demonstrated that 2-HBD is an effective PO inhibitor, and also revealed a few of the mechanistic details of the reaction. It may provide the basis for the design of effective, selective PO inhibitors and development of novel candidate insecticides.

Acknowledgments

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