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Inactivation Kinetics of Polyphenol Oxidase from Pupae of Blowfly (*Sarcophaga bullata*) in the Dimethyl Sulfoxide Solution

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Abstract The effects of dimethyl sulfoxide (DMSO) on the activity of polyphenol oxidase (PPO, EC 1.14.18.1) from blowfly pupae for the oxidation of L-3,4-dihydroxyphenylalanine were studied. The results showed that low concentrations of DMSO could lead to reversible inactivation to the enzyme. The IC_{50} value, the inactivator concentration leading to 50% activity lost, was estimated to be 2.35 M. Inactivation of the enzyme by DMSO was classified as mixed type. The kinetics of inactivation of PPO from blowfly pupae in the low concentrations of DMSO solution was studied using the kinetic method of the substrate reaction. The rate constants of inactivation were determined. The results show that k_{+0} was much larger than k'_{+0}, indicating that the free enzyme molecule was more fragile than the enzyme–substrate complex in the DMSO solution. It was suggested that the presence of the substrate offers marked protection of this enzyme against inactivation by DMSO.

Keywords Polyphenol oxidase · Blowfly pupae · Inactivation · Kinetics · Dimethyl sulfoxide

Abbreviation

DMSO	dimethyl sulfoxide
L-DOPA	L-3,4-dihydroxyphenylalanine
PPO	polyphenol oxidase
IC_{50}	the inactivator concentrations leading to 50% activity lost

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K_m the Michaelis–Menten constant
V_m the maximal velocity

Introduction

Polyphenol oxidase (PPO, EC 1.14.18.1), a copper-containing mixed-function oxidase, was widely distributed in nature and was responsible for the synthesis of melanin in animals and browning in plants [1–3]. This enzyme catalyzed the oxidation of *o*-diphenols to *o*-quinones. Quinones were highly reactive compounds and could polymerize spontaneously to form high-molecular-weight compounds or brown pigments (melanins) or react with amino acids and proteins that enhance the brown color produced [4, 5]. In insects, PPO was considered to be involved not only in melanin formation, but also in sclerotization of cuticles, wound healing, and defense reactions [6]. The inhibitors of PPO should have a range of applications. Many studies on the inhibition of PPO were carried out [7–10]. It was well known that PPO from plants and microorganisms could be inhibited by aromatic aldehydes and aromatic acids [11–14], tropolone [15], and flavonoids [16]. Because most inhibitors of PPO were hard to be dissolved in water, these compounds must be first dissolved in organic solvent, such as ethanol and dimethyl sulfoxide (DMSO) [8], and then tested for the effects on the enzyme activity. However, it was very important to decide if the enzyme was also affected by the organic solvent. In this investigation, it was found that the activity of PPO from blowfly pupae could be affected by DMSO, and the inactivation of the enzyme in DMSO solutions was shown to be reversible in low concentrations of DMSO. In this paper, we reported the effects of DMSO on the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA). And the inactivation kinetics and inactivation mechanism were also investigated.

Materials and Methods

PPO from pupae of blowfly (*Sarcophaga bullata*) was prepared and purified by a procedure involving ammonium sulfate fractionation and chromatography on diethylaminoethyl cellulose and Sephadex G-100 as described by Wang et al. [3]. The final preparation was homogeneous on polyacrylamide gel electrophoresis and high-performance liquid chromatography. The specific activity of the enzyme was 772 U/mg. One unit of enzymatic activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of dopachrome per minute from L-DOPA at 30°C. Absorption was recorded using a Beckman UV-650 spectrophotometer. DMSO and L-DOPA were purchased from Aldrich (USA). All other reagents were analytical grade. The water used was redistillated and ion-free.

PPO activity assay was performed as previously reported by Chen et al. [3, 17]. The assay system was 1.0 ml containing 1 mM of L-DOPA in 0.05 M Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8). Absorption and kinetic measurements were carried out using a Beckman UV-650 spectrophotometer.

Inactivation studies were performed by dissolving DMSO in an assay system. An enzyme aliquot (50 μl) was added to 1.0 ml of an assay system at 30°C, and the rate of substrate oxidation was monitored for 60 s after a 5-s lag period [8, 18].

The progress-of-substrate-reaction method previously described by Tsou [19, 20] was used for the study of the inactivation kinetics of the PPO of blowfly pupae. In this method, 50 μl of the enzyme (52.0 μg/ml) was added to 1.0 ml of reaction mixture

containing 1 mM L-DOPA in 0.05 M Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8) containing different concentrations of DMSO.

The fluorescence spectra were measured with a Hitachi 850 spectrophotometer. The enzyme (52.0 μg/ml) was dissolved in 1.0 ml of 0.05 M Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8) in different concentrations of DMSO and, at a constant temperature of 30°C, preincubated for 2 min before fluorescence spectra measurements with an excitation wavelength of 282.6 nm.

Results

Effect of DMSO on the PPO Activity for the Oxidation of L-DOPA

The effect of DMSO on the oxidation of L-DOPA by PPO of blowfly pupae was first studied. The relationship between residual enzyme activity and the concentrations of DMSO was shown in Fig. 1. The effect of DMSO on the enzyme activity was concentration-dependent. As the concentrations of DMSO increased, the residual enzyme activity rapidly decreased and reached complete inactivation when the concentration of DMSO was 5.6 M, indicating that the inactivation of the enzyme in DMSO solution was irreversible in high concentrations of DMSO. The IC_{50} value of DMSO was estimated to be 2.35 M.

Determination of the Kinetic Parameters of PPO from Blowfly Pupae

The kinetic behavior of PPO of blowfly pupae in catalyzing the oxidation of L-DOPA was studied. Under the condition employed in the present investigation, the oxidation reaction of L-DOPA by the PPO of blowfly pupae follows the Michaelis–Menten kinetics. The kinetic parameters for the enzyme obtained from a Lineweaver–Burk plot showed that K_m was equal to 1.838 mM and V_m was equal to 108.78 μM/min. In the DMSO concentration lower than 3.5 M, the inactivation of the enzyme was reversible, and the inactivation was

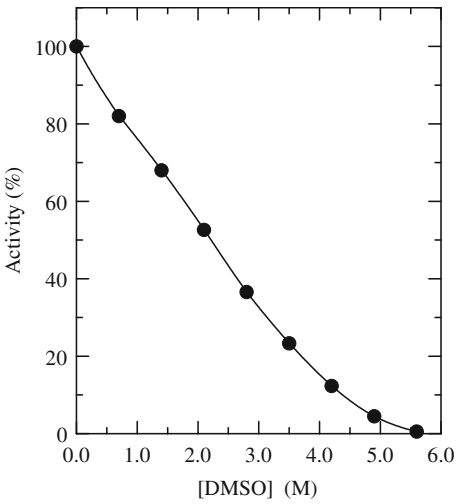
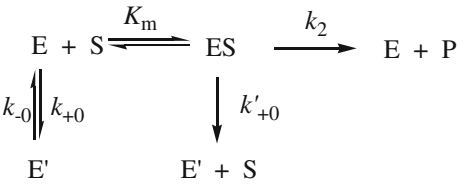


Fig. 1 Effect of DMSO on the activity of PPO from blowfly pupae for the oxidation of L-DOPA. Assay conditions: 250 μl system containing 0.05 M phosphate sodium buffer pH 6.8, 1.0 mM L-DOPA, and different concentrations of DMSO, 30°C

analyzed by the Lineweaver–Burk plots. The results illustrated in Fig. 2 showed that the inactivation of the enzyme was mixed type. Both K_m and V_m were changed. Increasing the concentration of DMSO, the K_m value increased and the V_m value decreased.

Establishment of the Kinetic Model of Inactivation of PPO in DMSO Solution

The temporal variation of the product concentration during the oxidation of L-DMSO by the PPO in the presence of different DMSO concentrations was shown in Fig. 3a. At each concentration of DMSO, the rate decreases with increasing time until a straight line was approached, the slope of which decreases with increasing DMSO concentration. The results suggest that, at DMSO concentration less than 3.5 M, denatured PPO still had partial residue activity (curves 1–5). This kinetic model can be written as follows:



where S, P, E and E' denote the substrate, product, and natural and inactivated enzyme, respectively; ES is the enzyme–substrate complex; k_{+0} and k_{-0} are the rate constants for forward and reverse inactivation of free enzyme, respectively; and k'_{+0} is the inactivation rate constant of the enzyme–substrate complex. As was the usual case $[S] \gg [E_0]$, product formation can be written as:

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

and

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

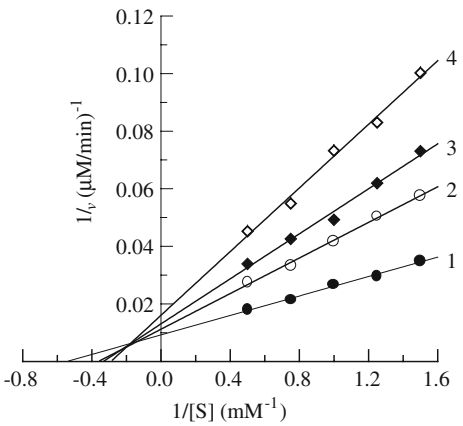


Fig. 2 Lineweaver–Burk plots for the oxidation of L-DOPA by PPO from blowfly pupae in different concentrations of DMSO. The DMSO concentration for *lines 1–4* was 0, 1.4, 2.1, 2.8, and 3.5 M, respectively. Conditions were the same as Fig. 1 except that the concentrations of L-DOPA was varied

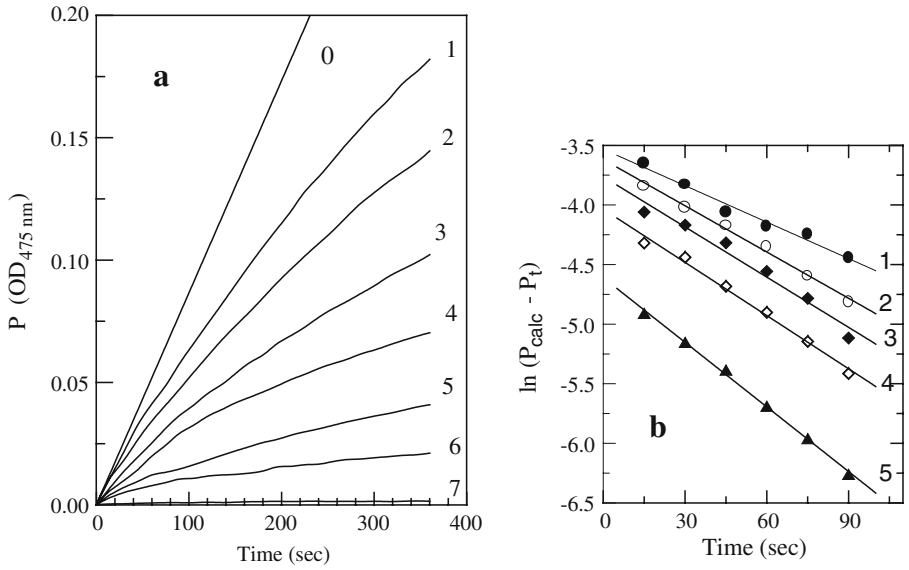


Fig. 3 Course of inactivation of PPO from blowfly pupae on incubation in different concentrations of DMSO. The assay conditions were the same as Fig. 2. **a** Substrate reaction course. The final DMSO concentrations for *curves 0–7* were 0, 0.7, 1.4, 2.1, 2.8, 3.5, 4.2, and 5.6 M, respectively. **b** Semilogarithmic plots of $\ln([P]_{\text{calc}}-[P]_t)$ against time *t*. Data were taken from *curves 1* to *4* in **a**

where $[P]_t$ is the concentration of the product formed at time *t*, which was the reaction time; *A* is the apparent forward rate constant of inactivation; [*S*] is the concentration of the substrate; and *v* is the initial rate of reaction in the absence of denaturant where $v=\frac{V_m\times[S]}{K_m+[S]}$. When *t* was sufficiently large, the curves become straight lines and the product concentration was written as $[P]_{\text{calc}}$:

$$[P]_{\text{calc}}=\frac{vk_{-0}}{A}\times t+\frac{v}{A^2}(A-k_{-0}). \tag{3}$$

Combining Eqs. 1 and 2 yields:

$$[P]_{\text{calc}}-[P]_t=\frac{v}{A^2}(A-k_{-0})\times e^{-A\times t}, \tag{4}$$

$$\ln\left([P]_{\text{calc}}-[P]_t\right)=-A\times t+\text{constant} \tag{5}$$

where $[P]_{\text{calc}}$ is the product concentration to be expected from the straight-line portions of the curves as calculated from Eq. 1 and $[P]_t$ is the product concentration actually observed at time *t*. Plots of $\ln([P]_{\text{calc}}-[P]_t)$ versus *t* give a series of straight lines at different concentrations of denaturant with slopes of $-A$ (Fig. 3b). The apparent forward rate constant *A* can be obtained from the slopes of the straight lines. A plot of $[P]_{\text{calc}}$ against time *t* gives a straight line with a slope of $\frac{vk_{-0}}{A}$. From the slope of the straight line, k_{-0} can be obtained.

Combining Eq. 2 and the Michaelis–Menten equation gives:

$$\frac{A}{v} = \frac{K_m}{V_m}(k_{+0} + k_{-0})\frac{1}{[S]} + \frac{k_{+0} + k_{-0}}{V_m} \tag{6}$$

A plot of A/v versus $1/[S]$ gives a straight line with $K_m(k_{+0} + k_{-0})/V_m$ and $\left(k'_{+0} + k_{-0}\right)/V_m$ as the slope and intercept, respectively. As K_m and V_m are known quantities from measurements of the substrate reaction in the absence of DMSO at different substrate concentrations and k_{-0} can be obtained from a suitable plot as above, the rate constants k_{+0} and k'_{+0} can be obtained from the slope and intercept of the straight line, respectively.

Measurement of Inactivation Rate Constant of PPO from Blowfly Pupae

The kinetic course of the oxidation reaction at different substrate concentrations in the presence of 2.1 M of DMSO was shown in Fig. 4a. When the time was sufficiently long, a straight line was approached at each concentration of substrate. Both the initial rate and the slope of the asymptote increased with increasing substrate concentration (Fig. 4a). From Eq. 5, plots of $\ln([P]_{calc} - [P]_t)$ versus t gave a series of straight lines at

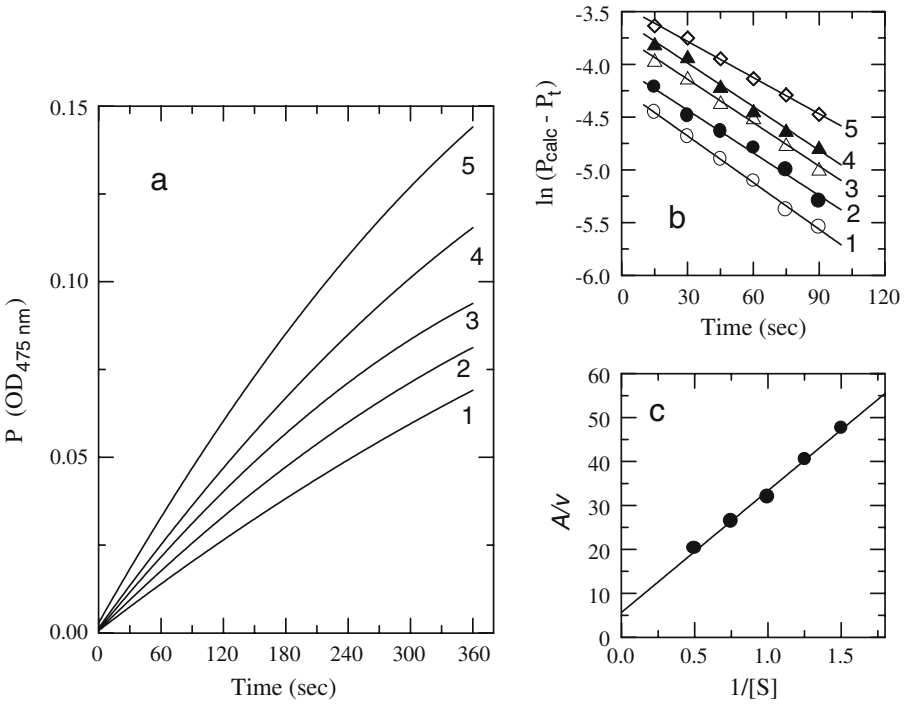


Fig. 4 Determination of the inactivation rate constants for inactivation of PPO from blowfly pupae in 2.1 M of DMSO solution. **a** Substrate reaction courses of the enzyme in the presence of 2.1 M DMSO. *Curves 1–5* are progress curves with 0.667, 0.8, 1.0, 1.5, and 2 mM of substrate, respectively. The other conditions are the same. **b** Semilogarithmic plot of $\ln([P]_{calc} - [P]_t)$ against time t for data given in **a**. The lines are numbered as for **a**. **c** Plot of A/v versus $1/[S]$. The A were obtained from the slopes of the straight lines in **b**

different concentrations of substrate whose slopes were equal to the apparent forward rate constant A (Fig. 4b). Since K_m and V_m were known quantities, the values of $(k_{+0}+k_{-0})$ and k'_{+0} could be obtained from the slope and the intercept of the straight line in Fig. 4c, a plot of A/v versus $1/[S]$ according to Eq. 6. From Eq. 3, a plot of $[P]_{calc}$ against time t gave a straight line with a slope of $\frac{vk_{-0}}{A}$. From the slope of the straight line, k_{-0} could be obtained. From the above values, the inactivation rate constants k_{+0}, k_{-0}, and k'_{+0} were obtained. The above results are shown in Table 1. Similarly, the inactivation rate constants of mushroom tyrosinase at other DMSO concentrations were also obtained and the results obtained were summed in Table 1.

Fluorescence Emission Spectra of PPO from Blowfly Pupae in DMSO Solutions

The fluorescence emission spectra of the enzyme in different concentrations of DMSO were shown in Fig. 5. The emission peak of the native enzyme was at 334.8 nm; it might contain contributions from both Trp residues and Tyr residues. Increasing the DMSO concentration caused the fluorescence emission intensity to be markedly increased and the emission peak (at 334.8 nm) to be red-shifted a little. When the concentration of DMSO reached 2.8 M, the fluorescence intensity from 13.0 increased to 18.5, increasing by 42.3%. And the red shift of the emission maximum was only 5.2 nm. The result indicated that when the DMSO binds to the enzyme molecule, it can induce the enzyme conformation to change and then results in the inactivation of the enzyme.

Discussion

PPO was considered to be involved in melanin formation. In insects, it was a key enzyme involved in sclerotization of cuticles, wound healing, and defense reactions [6]. So, it was very important to research its inhibitors in order to find the insecticide. Many inhibitors of the PPO were hard to be dissolved in water; they must first be dissolved in organic solvent, such as ethanol and DMSO, and then tested for inhibition on enzyme activity. The capacity to accept hydrogen bonds and the relatively small and compact structure of DMSO makes it easier to associate with water, proteins, carbohydrates, nucleic acid, ionic substances, and so on [8]. Just because of this, DMSO was added to the reaction system to increase the solubility of organic inhibitors [21]. But, the interaction between DMSO and this enzyme has not been reported. Hence, it aroused us to detect if the enzyme was also affected by this organic solvent. In addition, enzymes and proteins in organic solvents received expanding attention in the past decade, and some novel properties were reported while enzymes work in organic

Table 1 Microscopic rate constants of the inactivation of PPO from blowfly pupae in DMSO solutions.

[DMSO] (mol/L)	Residual activity (%)	Rate constants (s^{-1})			Fluorescence intensity (%)
		k_{+0}	k_{-0}	k'_{+0}	
0.0	100				100.0
1.4	68.0	0.0468	0.00977	0.0188	113.8
2.1	52.6	0.0924	0.00871	0.0293	123.1
2.8	36.6	0.1248	0.00693	0.0554	142.3

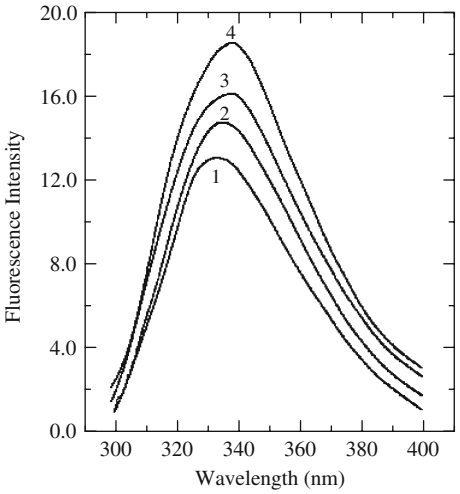


Fig. 5 Fluorescence emission spectra of PPO from blowfly pupae inactivated in the DMSO solutions. Forty microliters of enzyme was incubated in 1 ml of 0.05 M phosphate sodium buffer pH 6.8 containing different concentrations of DMSO at 30°C for 2 min before determination of the fluorescence spectra. The excitation wavelength was 284.2 nm. The enzyme concentration was 25.0 μg/ml. The DMSO concentration in the inactivation mixture for *curves 1–4* were 0, 1.4, 2.1, and 2.8 M, respectively

solvents, which benefit both biotechnology and pharmaceutical industry. In this paper, we not only used the substrate reaction kinetic method to analyze the inactivation kinetics of PPO from blowfly pupae and obtain the kinetics constants, but also elucidated the relationship between the changes of conformation and function of the enzyme and the concentrations of DMSO. The results showed that, in the low concentration of DMSO, the enzyme activity inhibited reversibly, 0.46 M (3.3%) of DMSO can result in the enzyme activity losing by 5%, and increasing its concentrations, the enzyme activity exponentially decreased. When the concentration of DMSO reached 2.2 M (15.7%), the enzyme activity lost 50%. The results listed in Table 1 show that k_{+0} was much larger than k'_{+0}, indicating that the free enzyme molecule was more fragile than the enzyme–substrate complex in the DMSO solution.

Accompanying the loss of activity that resulted from the increasing concentration of DMSO, the fluorescence intensity of the enzyme in DMSO solution increased and the emission peak was red-shifted indistinctively. The change of the fluorescence intensity of PPO from blowfly pupae in DMSO solution was the same as that of mushroom tyrosinase in the DMSO [8], but very different from that of prawn NAGase [20] whose fluorescence intensity decrease with increasing the dioxane concentration. Maybe it was because of the polarity difference between DMSO and dioxane. Our results showed that DMSO could cause the changes of pH or dielectric constant and would modify the nature and the number of noncovalent interaction, which could change the microenvironments of Trp and Tyr residues in the enzyme molecules, and make the enzyme conformation unfold, and the activity loss.

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References

1. Yoruk, R., & Marshall, M. R. (2003). Physicochemical properties and function of plant polyphenoloxidase: a review. *Journal of Food Biochemistry, 27*(5), 361–422. doi:10.1111/j.1745-4514.2003.tb00289.x.

2. Xue, C. B., Lou, W. C., Jiang, L., Xie, X. Y., Xiao, T., & Yan, L. (2007). Inhibition kinetics of cabbage butterfly (*Pieris rapae* L.) larvae phenoloxidase activity by 3-hydroxy-4-methoxybenzaldehyde thiosemicarbazone. *Applied Biochemistry and Biotechnology, 143*(2), 101–114. doi:10.1007/s12010-007-8007-0.
3. Wang, Q., Chen, Q. X., Huang, X. H., Ke, L. N., Shi, Y., & Wang, J. (2004). Studies on the enzymatic characterization and functional groups of polyphenoloxidase form pupae of blowfly (*Sarcophaga bullata*). *Biochemistry (Moscow), 69*(8), 918–920. doi:10.1023/B:BIRY.0000040225.33267.26.
4. Xue, C. B., Luo, W. C., Chen, Q. X., Ma, D. Y., & Wang, Q. (2008). Inhibitory effects of 2-hydroxybenzaldehyde on the activity of phenoloxidase form *Pieris rapae* (Lepidoptera) Larvae. *Indian Journal of Biochemistry & Biophysics, 45*, 184–191.
5. Kanade, S. R., Suhas, V. L., Chandra, N., & Gowda, L. R. (2007). Functional interaction of diphenols with polyphenoloxidase. Molecular determinants of substrate/inhibitor specificity. *Journal of FEBS, 274* (16), 4177–4187. doi:10.1111/j.1742-4658.2007.05944.x.
6. Fujimoto, K., Masuda, K. I., Asada, N., & Ohnishi, E. (1993). Purification and characterization of prophenoloxidases from pupae of *Drosophila melanogaster*. *Journal of Biochemistry, 113*, 285–291.
7. Yoruk, R., Hogsette, J. A., Rolle, R. S., & Marshall, M. R. (2003). Apple polyphenol oxidase inhibitor(s) from common house fly (*Musca domestica* L.). *Journal of Food Science, 68*(6), 1942–1947. doi:10.1111/j.1365-2621.2003.tb06998.x.
8. Chen, Q. X., Liu, X. D., & Huang, H. (2003). Inactivation kinetics of mushroom tyrosinase in the dimethyl sulfoxide solution. *Biochemistry (Moscow), 68*(6), 644–649. doi:10.1023/A:1024665709631.
9. Han, P., Chen, C. Q., Zhang, C. L., Song, K. K., Zhou, H. T., & Chen, Q. X. (2008). Inhibitory effects of 4-chlorosalicylic acid on mushroom tyrosinase and its antimicrobial activities. *Food Chemistry, 107*, 797–803. doi:10.1016/j.foodchem.2007.08.084.
10. Wang, Q., Ke, L. N., Xue, C. B., Luo, W. C., & Chen, Q. X. (2007). Inhibitory kinetics of *p*-substituted benzaldehydes on polyphenol oxidase from the fifth instar of *Pieres Rapae* L. *Tsinghua Science & Technology, 12*, 400–404. doi:10.1016/S1007-0214(07)70060-3.
11. Robert, C., Rouch, C., & Cadet, F. (1997). Inhibition of palmito (*Acanthophoenix rubra*) polyphenol oxidase by carboxylic acids. *Food Chemistry, 59*, 355–360. doi:10.1016/S0308-8146(96)00179-3.
12. Chen, Q. X., Lin, J. F., & Song, K. K. (2007). Development of tyrosinase inhibitors. *Journal of Xiamen University, 46*(2), 274–282. (in Chinese).
13. Chen, Q. X., Song, K. K., Qiu, L., Liu, X. D., Huang, H., & Guo, H. Y. (2005). Inhibitory effects on mushroom tyrosinase by *p*-alkoxybenzoic acids. *Food Chemistry, 91*, 269–274. doi:10.1016/j.foodchem.2004.01.078.
14. Huang, X. H., Chen, Q. X., Wang, Q., Song, K. K., Wang, J., Sha, L., et al. (2006). Inhibition of alkylbenzoic acids on the activity of mushroom tyrosinase. *Food Chemistry, 94*, 1–6. doi:10.1016/j.foodchem.2004.09.008.
15. Saniewski, M., Saniewska, A., & Kanlayanarat, S. (2007). Biological activities of tropolone and hinokitiol: the tools in plant physiology and their practical use. Proceedings of the International Conference on Quality Management in Supply Chains of Ornamentals. *Acta Horticulturae, 755*, 133–142.
16. Xie, L. P., Chen, Q. X., Huang, H., Wang, H. Z., & Zhang, R. Q. (2003). Inhibitory effects of some flavonoids on the activity of mushroom tyrosinase. *Biochemistry (Moscow), 68*(4), 487–491. doi:10.1023/A:1023620501702.
17. Xie, J. J., Song, K. K., Qiu, L., He, Q., Huang, H., & Chen, Q. X. (2007). Inhibitory effects of substrate analogs on enzyme activity and substrate specificities of mushroom tyrosinase. *Food Chemistry, 103*, 1075–1079. doi:10.1016/j.foodchem.2006.04.030.
18. Xie, J. J., Chen, Q. X., Wang, Q., Song, K. K., & Qiu, L. (2007). Activation kinetics of cetylpyridinium chloride on the prophenol oxidase from pupae of blowfly (*Sarcophaga bullata*). *Pesticide Biochemistry and Physiology, 87*, 9–13. doi:10.1016/j.pestbp.2006.03.004.
19. Tsou, C. L. (1988). Kinetics of substrate reaction during irreversible modification of enzyme activity. *Advances in Enzymology and Related Areas of Molecular Biology, 61*, 381–436. doi:10.1002/9780470123072.ch7.
20. Xie, J. J., Chen, C. Q., Wang, Q., Zhang, J. P., Lin, J. C., & Chen, Q. X. (2009). Inactivation kinetics of β-*N*-acetyl-D-glucosaminidase from green crab (*Scylla serrata*) in dioxane solution. *Journal of Biomolecular Structure & Dynamic, 26*(4), 509–515.
21. Qiu, L., Chen, Q. H., Zhuang, J. X., Zhong, X., Zhou, J. J., Guo, Y. J., et al. (2009). Inhibitory effects of α-cyano-4-hydroxycinnamic acid on the activity of mushroom tyrosinase. *Food Chemistry, 112*, 609–613. doi:10.1016/j.foodchem.2008.06.021.