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Inactivation Kinetics of Mushroom Tyrosinase in the Dimethyl Sulfoxide Solution

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Abstract—Mushroom tyrosinase (EC 1.14.18.1) is a kind of copper-containing oxidase that catalyzes both the hydroxylation of tyrosine into *o*-diphenols and the oxidation of *o*-diphenols into *o*-quinones and then forms brown or black pigments. In the present paper, the effects of dimethyl sulfoxide on the enzyme activity for the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) have been studied. The results show that low concentrations of dimethyl sulfoxide (DMSO) can lead to reversible inactivation of the enzyme, and the IC_{50} is estimated to be 2.45 M. Inactivation of the enzyme by DMSO is classified as mixed type. The kinetics of inactivation of mushroom tyrosinase at low concentrations of DMSO solution has been studied using the kinetic method of the substrate reaction. The rate constants of inactivation have been determined. The results show the free enzyme molecule is more fragile than the enzyme–substrate complex in the DMSO solution. It is suggested that the presence of the substrate offers marked protection of this enzyme against inactivation by DMSO.

Key words: mushroom tyrosinase, inactivation, kinetics, dimethyl sulfoxide

Tyrosinase (EC 1.14.18.1), a copper-containing mixed-function oxidase, is widely distributed in nature and is responsible for the synthesis of melanin in animals and for browning in plants. This enzyme catalyzes both the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones. Quinones are highly reactive compounds and can 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 [1-4]. In some vegetables and fruits, tyrosinase is responsible for browning and is considered deleterious to the color quality of plant-derived foods and beverages. The unfavorable browning of raw fruits, vegetables, and beverages is a major problem in the food industry and is believed to be one of the main causes of quality loss during post harvest handling and processing. Because of the undesirable effects of enzymatic browning, tyrosinase inhibitors should have a range of applications. It is well known that mushroom tyrosinase can be inhibited by aromatic aldehydes and acids [5, 6], tropolone [7], and kojic acid [8]. 4-Hexylresorcinol has been claimed to be the most effective inhibitor used in the food industry, and it has been recognized as safe for use in the prevention of shrimp melanosis [9, 10] and for browning control of fresh and hot-air-dried apple slices as well as potatoes, avocados, and apple and grape juices [11]. Although a large number of naturally occurring tyrosinase inhibitors have already been described, either their individual activity is not potent enough to be considered of practical use or safety regulations concerning food additives limit their *in vivo* use. There is, therefore, a constant search for tyrosinase inhibitors that can be obtained then by laboratory synthesis [12] or extraction from plants [13, 14]. Recently, we found some common flavonoids can strongly inhibit mushroom tyrosinase for the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) [15]. Because these compounds are hard to dissolve in water, these compounds must be first dissolved in organic solvent, such as ethanol and dimethyl sulfoxide, and then tested for the effects on the enzyme activity. However, it is very important to decide if the enzyme is also affected by the organic solvent. In our investigation, we found that mushroom tyrosinase activity can be affected by dimethyl sulfoxide, and the inactivation of the enzyme in dimethyl sulfoxide solutions was showed to be reversible in low concentration of dimethyl sulfoxide. In this paper we report the

Abbreviations: DMSO) dimethyl sulfoxide; L-DOPA) L-3,4-dihydroxyphenylalanine; Na_2HPO_4-NaH_2PO_4) disodium hydrogen phosphate-sodium dihydrogen phosphate buffer.

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effects of dimethyl sulfoxide on the oxidation of L-DOPA. The inactivation kinetics and inactivation mechanism were also investigated.

MATERIALS AND METHODS

Mushroom tyrosinase (EC 1.14.18.1) was the product of Sigma (USA). The specific activity of the enzyme is 6680 U/mg. One unit (U) of enzymatic activity was defined as the amount of enzyme increasing absorbance by 0.001 at 475 nm at 25°C. Dimethyl sulfoxide (DMSO) and L-3,4-dihydroxyphenylalanine (L-DOPA) was purchased from Aldrich (USA). All other reagents were of analytical grade. The water used was re-distillated and ion-free.

The tyrosinase assay was performed as previously reported [16] and the activity was determined at 30°C by following the increasing absorbance at 475 nm accompanying the oxidation of the substrate (L-DOPA) with the molar absorption coefficient of 3700 $M^{-1}\cdot cm^{-1}$ [17]. The assay system was 250 µl 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 Spectra MAX plus Microplate spectrophotometer.

Inactivation studies were performed by dissolving DMSO in an assay system. An enzyme aliquot (50 µl) was added to 250 µl of an assay system at 30°C, and the rate of substrate oxidation was monitored for 30 sec after a 5-sec lag period.

The progress-of-substrate-reaction method as previously described [18-21] was used to study the inactivation kinetics of mushroom tyrosinase in DMSO solutions. In this method, 50 µl of mushroom tyrosinase (20 µg/ml in 0.05 M Na_2HPO_4-NaH_2PO_4 buffer, pH 6.8) was added to 200 µl of the assay system. The final reaction condition was that the 250 µl system contained different concentrations of L-DOPA in 0.05 M Na_2HPO_4-NaH_2PO_4 buffer (pH 6.8) with different concentrations of DMSO. The substrate reaction progress curve was analyzed to obtain the reaction rate constants. The final concentration of mushroom tyrosinase was 0.0333 µM. The reaction was carried out at a constant temperature of 30°C.

The fluorescence spectra were measured with a Hitachi 850 spectrophotometer (Japan). Mushroom tyrosinase (40 µg) was dissolved in 1 ml of 0.05 M Na_2HPO_4-NaH_2PO_4 buffer (pH 6.8) with 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 284.6 nm.

RESULTS

Effect of DMSO on mushroom tyrosinase activity for the oxidation of L-DOPA. The effect of DMSO on the oxidation of L-DOPA by mushroom tyrosinase was first studied. The relationship between residual enzyme activity and the concentrations of DMSO was shown in Fig. 1. The effect of DMSO on mushroom tyrosinase was concentration dependent. As the concentrations of DMSO increased, the residual enzyme activity rapidly decreased. The DMSO concentration leading to 50% activity lost (IC_{50}) was estimated to be 2.45 M.

Determination of the kinetic parameters of mushroom tyrosinase. The kinetic behavior of mushroom tyrosinase in catalyzing the oxidation of L-DOPA was studied. Under the condition employed in the present investigation, the oxidation reaction of L-DOPA by mushroom tyrosinase follows Michaelis–Menten kinetics. The kinetic parameters for mushroom tyrosinase obtained from a Lineweaver–Burk plot showed that K_m was equal to 0.549 ± 0.020 mM and V_m was equal to 51.67 ± 1.50 µM/min. Tyrosinase is known to catalyze a reaction between two substrates, a phenolic compound (L-DOPA) and oxygen, but the assay was carried out in air-saturated aqueous solutions. Therefore, Michaelis constant (K_m) and maximum velocity (V_m) values determined under these conditions were only apparent, and the effect of oxygen concentration on these parameters would be unknown. At lower than 3.5 M of DMSO, the inactivation of the enzyme was reversible, and the inactivation was analyzed by Lineweaver–Burk plots. The results illustrated in Fig. 2

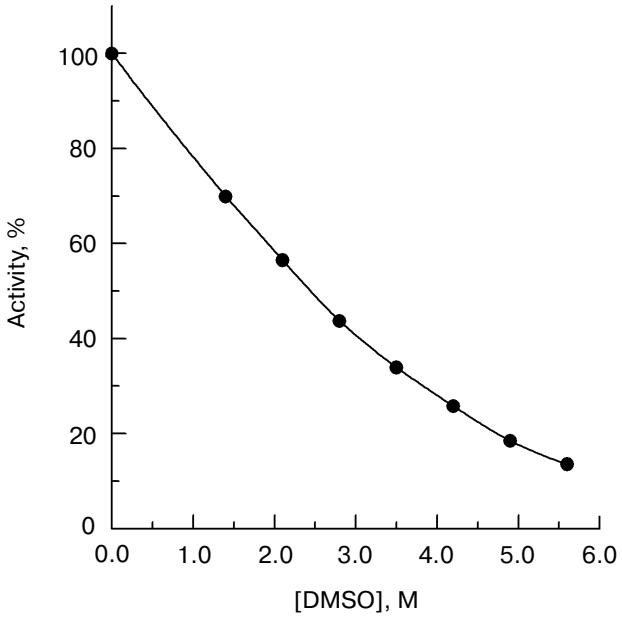


Fig. 1. Effect of dimethyl sulfoxide on the activity of mushroom tyrosinase for the oxidation of L-DOPA. Assay conditions: 250 µl system containing 0.05 M sodium phosphate buffer, pH 6.8, 1 mM L-DOPA, and different concentrations of dimethyl sulfoxide, 30°C for 1 min. The enzyme concentration was 0.0333 µM.

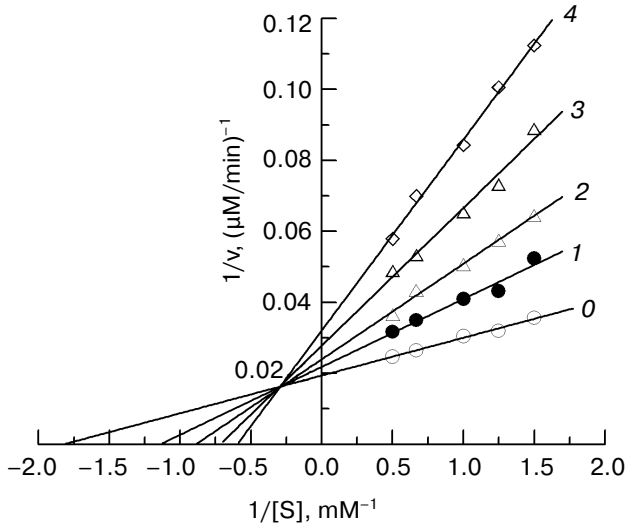
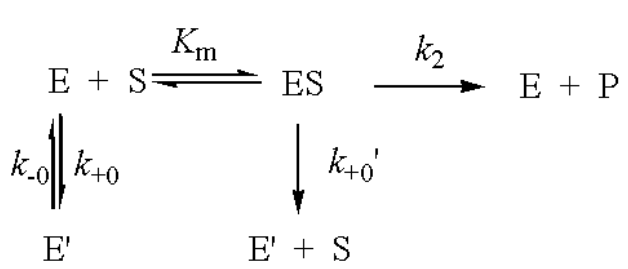


Fig. 2. Lineweaver–Burk plots for the oxidation of L-DOPA by mushroom tyrosinase at different concentrations of dimethyl sulfoxide. The DMSO concentration for lines *0-4* was 0, 1.4, 2.1, 2.8, and 3.5 M, respectively. The assay conditions were the same as in Fig. 1 except that the concentrations of L-DOPA were variable.

showed that the inactivation of mushroom tyrosinase was of mixed type. Increasing the concentration of DMSO, the K_m value increased and the V_m value decreased.

Inactivation rate constants of mushroom tyrosinase in DMSO solutions. The progress-of-substrate-reaction method previously described by Tsou [22] was used for the study of the inactivation kinetics of mushroom tyrosinase. In this method, 50 μl of 20 μg/ml tyrosinase was added to 0.25 ml of reaction mixture containing 1 mM L-DOPA in 0.05 M Na_2HPO_4-NaH_2PO_4 buffer, pH 6.8, with different concentrations of DMSO. The substrate reaction progress curve (Fig. 3) was analyzed to obtain the rate constants as detailed below. The time course of the oxidation of the substrate in the presence of different DMSO concentrations showed that, at lower than 3.5 M of DMSO, the rate decreased with increasing time until a straight line was approached. The results showed that the inactivation was a reversible reaction at lower than 3.5 M of DMSO with fractional residue activity. When the concentration of DMSO was higher than 4.2 M, the inactivation was an irreversible course. This can be written as the following scheme:



where S, P, E, and E′ denote substrate, product, and the native and inactivated enzyme, respectively; ES is the enzyme–substrate complex; k_{+0} and k_{-0} are rate constants for the forward and reverse inactivation of free enzyme, respectively, and k_{+0}' is inactivation rate constant of the enzyme–substrate complex. As is usually the case [S] >> [E_0], the product formation can be written as:

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

and

$$A = \frac{k_{+0}K_m + k_{+0}'[S]}{K_m + [S]} + k_{-0} \; , \qquad (2)$$

where $[P]_t$ is the concentration of the product formed at time t, which is the reaction time; A is the apparent forward rate constant of inactivation, respectively; [S] is the concentration of the substrate; and v is the initial rate of reaction in the absence of denaturant, where

$$v = \frac{V_m \cdot [S]}{K_m + [S]}$$

When t is sufficiently large, the curves become straight lines and the product concentration is written as $[P]_{calc}$:

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

Combining Eqs. (1) and (3) yields:

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

$$\ln([P]_{calc} - [P]_t) = -A \cdot t + \text{constant} \, , \qquad (5)$$

where $[P]_{calc}$ is the product concentration to be 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 $\ln([P]_{calc} - [P]_t)$ versus t give a series of straight lines at different concentrations of denaturant with slopes of $-A$. The apparent forward rate constant A can be obtained from such graphs. From Eq. (3), a plot of $[P]_{calc}$ 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:

$$\frac{A}{v} = \frac{K_m}{V_m}(k_{+0} + k_{-0})\frac{1}{[S]} + \frac{k'_{+0} + k_{-0}}{V_m} \quad . \qquad (6)$$

A plot of A/v versus 1/[S] gives a straight line with $K_m(k_{+0} + k_{-0})/V_m$ and $(k_{+0}' + k_{-0})/V_m$ 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 mushroom tyrosinase in DMSO solutions. The temporal variation of the product concentration during the substrate hydrolysis in the presence of different DMSO concentrations is shown in Fig. 3. At each concentration of DMSO, the rate decreases with increasing time until a straight line is approached, the slope of which decreases with increasing DMSO concentration. When the concentration of DMSO is above 4.2 M, the enzyme is inactivated very rapidly and the inactivation is irreversible. The results suggest that at DMSO concentration less than 4.2 M, denatured tyrosinase still had partial residue activity (curves *1-5*). According to Eq. (5), plots of $\ln([P]_{calc} - [P]_t)$ versus t give a series of straight lines shown in Fig. 3b. From the slopes of the straight line, the apparent forward rate constant of inactivation, A, can be obtained.

The kinetic course of the oxidation reaction at different substrate concentrations in the presence of 2.1 M DMSO is shown in Fig. 4a. In the presence of 2.1 M of DMSO, when the time is sufficiently long, a straight line is approached at each concentration of substrate. Both the initial rate and the slope of the asymptote increase with increasing substrate concentration (Fig. 4a). From Eq. (5), plots of $\ln([P]_{calc} - [P]_t)$ versus t give a series of straight lines at different concentrations of substrate, whose slopes are equal to the apparent forward rate constant A (Fig. 4b). Since K_m and V_m are quantities known, the values of $(k_{+0} + k_{-0})$ and k_{+0}' can 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 gives a straight line with a slope of $v \cdot k_{-0}/A$. From the slope of the straight line, k_{-0} can 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 the table. Similarly, the inactivation rate constants of mushroom tyrosinase at other DMSO concentrations were also obtained (the table).

Fluorescence emission spectra of mushroom tyrosinase in DMSO solutions. The fluorescence emission spectra of mushroom tyrosinase in different concentrations of DMSO are shown in Fig. 5. The emission peak of the native enzyme is at 335 nm; it may contain con-

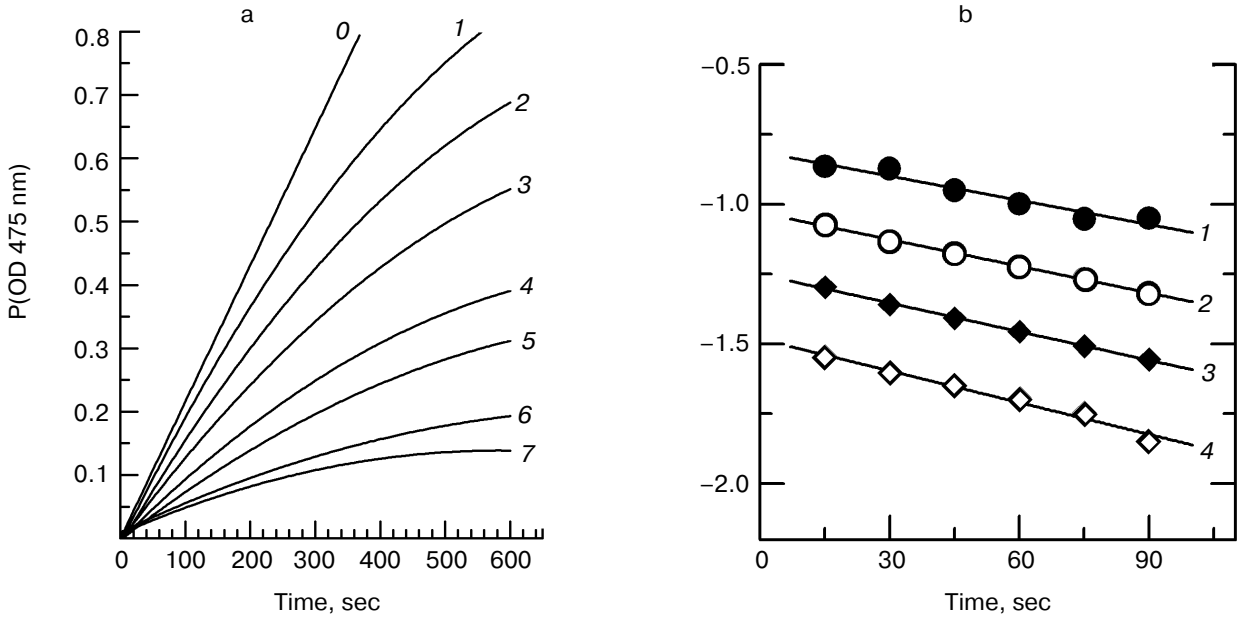


Fig. 3. Course of inhibition of mushroom tyrosinase on incubation in different concentrations of dimethyl sulfoxide. The assay conditions were the same as in Fig. 1. a) Substrate reaction course. The final DMSO concentrations for curves *0-7* were 0, 1.4, 2.1, 2.8, 3.5, 4.2, 4.9, and 5.6 M, respectively. b) Semilogarithmic plots of $\ln([P]_{calc} - [P]_t)$ against time. Data were taken from curves *1-4* in Fig. 3a.

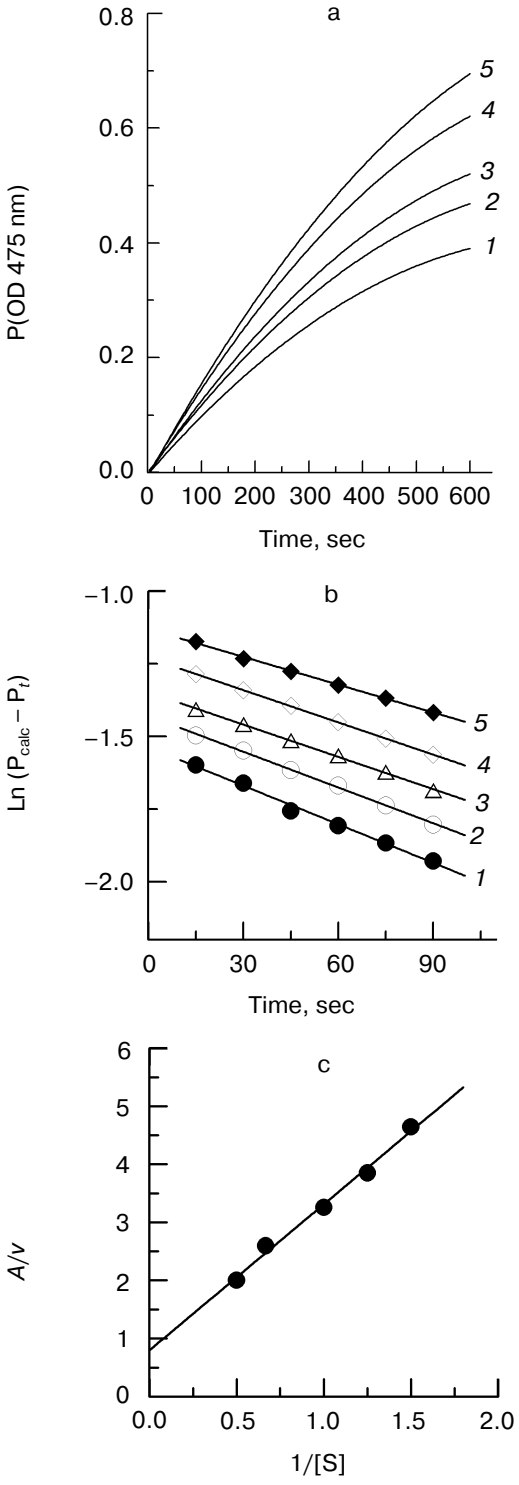


Fig. 4. Determination of the inactivation rate constants for inactivation of mushroom tyrosinase in 2.1 M DMSO solution. a) Substrate reaction courses of the enzyme in the presence of 2.1 M DMSO. Curves *1-5* are progress curves with 0.667, 0.8, 1.0, 1.5, and 2 mM of substrate, respectively. The other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

tributions from both Trp and Tyr residues. When the concentration of DMSO reached 3.5 M, the fluorescence emission intensity increased by 40% and the red shift of the emission maximum was 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

It was reported that mushroom tyrosinase can be inhibited by some compounds. Some of those compounds were hard to dissolve in water. They must first be dissolved in organic solvent, such as ethanol and dimethyl sulfoxide, and then tested for the inhibition on the enzyme activity. The capacity to accept hydrogen bonds and the relatively small and compact structure of DMSO make it easier to associate with water, proteins, carbohydrates, nucleic acid, ionic substances, and so on. Just because of this, DMSO was added to the reaction system to increase the solubility of the organic inhibitors. But, the interaction between DMSO and the

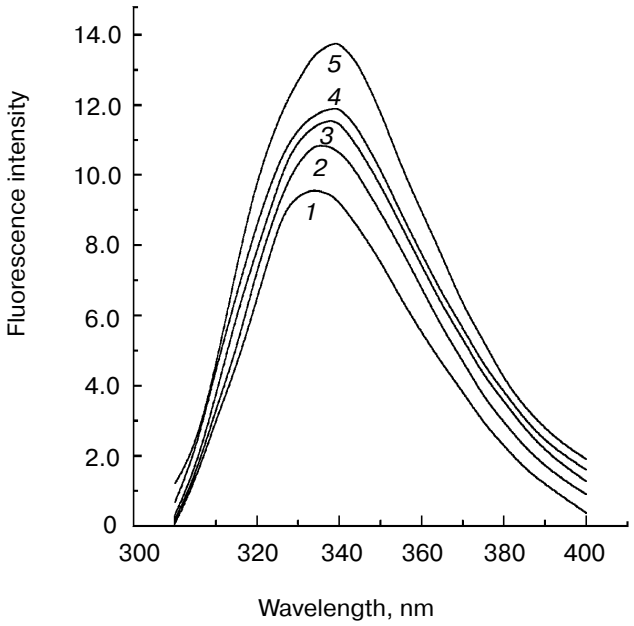


Fig. 5. Fluorescence emission spectra of mushroom tyrosinase inactivated in the DMSO solutions. Enzyme (40 μl) was incubated in 1 ml of 0.05 M sodium phosphate buffer, pH 6.8, containing different concentrations of DMSO at 30°C for 2 min before determination of the fluorescence spectra. The excitation wavelength was 284.2 nm. The enzyme concentration was 0.3333 μM. The DMSO concentration in the inactivation mixture for curves *1-5* were 0, 1.4, 2.1, 2.8, and 3.5 M, respectively.

Microscopic rate constants of the inactivation of mushroom tyrosinase in DMSO solutions

[DMSO], M	Rate constants × 10^3, sec^{-1}			Residual activity, %	Fluorescence intensity, %
	k_{+0}	k_{-0}	k_{+0}'		
0				100.0	100.0
1.4	12.237	1.785	0.732	69.86	112.6
2.1	13.151	1.439	1.103	56.50	120.0
2.8	15.526	1.216	1.970	43.70	125.3
3.5	16.722	0.744	2.946	33.89	143.2

enzyme has not been reported. Hence, it motivated us to determine if the enzyme is also affected by this organic solvent. In addition, enzymes and proteins in organic solvents have received expanding attention in the past decade, and some novel properties have been reported while enzymes work in organic solvents, which benefit both biotechnology and pharmaceutical industry. In this paper, we not only used the substrate reaction kinetic method to analyze the inhibitory kinetics of mushroom tyrosinase and obtain the kinetics constants, but also elucidated the relationship between the changes in conformation and function of mushroom tyrosinase and the concentrations of DMSO. The results showed that, in the low concentration of DMSO, the enzyme activity was inhibited reversibly, 0.46 M (3.3%) of DMSO can result in the enzyme losing activity by 5%, and increasing its concentrations, the enzyme activity decreased exponentially. When the concentration of DMSO reached to 17.5% (2.45 M), the enzyme lost 50% of its activity. The results listed in the table show that k_{+0} is much larger than k_{+0}', indicating the free enzyme molecule is more fragile than the enzyme–substrate complex in the DMSO solution.

Accompanying the loss of activity resulting from rising concentration of DMSO, the fluorescence intensity increased and the emission peak was red-shifted. This may be it is because the adding of DMSO causes the change of pH or dielectric constant, or the presence of organic reagent, DMSO, could modify the nature and the number of noncovalent interactions, so the microenvironments of tyrosinase change and then its conformation is affected, and its activity decreases.

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