Partial Purification and Characterization of Polyphenoloxidase from Culinary-Medicinal Royal Sun Mushroom (the Himematsutake), *Agaricus brasiliensis* S. Wasser et al. (Agaricomycetideae)

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ABSTRACT: The Royal Sun mushroom, the Himematsutake culinary-medicinal mushroom, *Agaricus brasiliensis* has several polyphenoloxidase activities in a broad sense. Here we report the partial purification of tyrosinase-type polyphenoloxidase (PPO). PPO is purified from *A. brasiliensis* without browning using a two-phase partitioning with Triton X-114 and ammonium sulfate fractionation. Partially denaturing SDS-PAGE (sodium dodecyl sulfate-polyacrylamide electrophoresis) staining with L-3,4-dihydroxyphenylalanine was performed and the indicated molecular sizes were approximately 70 kDa and 45 kDa. The purified enzyme is in its latent state and can be activated maximally in the presence of 1.6 mM sodium dodecyl sulfate (SDS). This enzyme catalyzes two distinct reactions, monophenolase and diphenolase activity, and the monophenolase activity showed a lag time typical of polyphenoloxidase. The K_m value for 4-*tert*-butylcatechol was quite similar in the presence and absence of SDS, but the apparent V_{max} value was increased 2.0-fold by SDS. Mimosine was a typical competitive inhibitor with K_i values of 138.2 μM and 281.0 μM n the presence and absence of SDS, respectively.

KEY WORDS: medicinal mushrooms, Royal Sun mushroom, *Agaricus brasiliensis*, Triton X-114, polyphenoloxidase, tyrosinase, SDS activation

ABBREVIATIONS: DTT: dithiothreitol; **L-DOPA:** L-3,4-dihydroxyphenylalanine; **PPO:** polyphenoloxidase; **SDS:** sodium dodecyl sulfate; **SDS-PAGE:** sodium dodecyl sulfate-polyacrylamide electrophoresis; **TBC:** 4-*tert*-butylcatechol; **TX-114:** Triton X-114

I. INTRODUTION

Extracts from fruiting bodies and mycelia of fungi are effective for preserving human health, such as prevention, treatment, and recovery from disease. In particular, it is known in China, Japan, Russia, Canada, Mexico, and the United States that medicinal mushrooms can be used in cancer treatment, which is described in ancient books in China and Japan. A main component of the extracts is beta-glucan, which shows a biological response-modifying effect. The Royal Sun mushroom (the Himematsutake), *Agaricus brasiliensis* S. Wasser et al. (=*A. blazei* sensu Heinem.) (Agaricaceae, Agaricomycetideae, Basidiomycetes) is an edible species that is found in Brazil and cultured in Indonesia, China, Korea, Japan, and the United States. It has been reported recently that its chemical components have antitumor and biological response-modifying effects.[1,2] Our group previously showed that polysaccharides, mainly (1-6)-beta-D-glucan, found in the supernatant of hot water and sodium hydroxide extracts from *A. brasiliensis*, mediate the immunological response and exhibit antitumor activity.[3] To test the possibility that the pharmacologic activity of *A. brasiliensis* is influenced by the culture condition, we compared two *A. brasiliensis* fruiting bodies, which originate from the same strain and were

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processed in the same manner but cultured under different conditions (i.e., indoors versus field), in terms of their chemical components and biological activity. Remarkable differences were eventually found by two-dimensional gel electrophoresis of their protein fraction and in their enzyme activities, especially for tyrosinases, polyphenoloxydases (PPO), laccases, peroxidases, and beta-glucanases.[4]

The binuclear enzymes, PPO and tyrosinase (EC 1.14.18.1), were found in the middle of the 19th century and were partially purified at the beginning of the 20th century. These enzymes exist widely in microorganisms, plants, and animals, and have been purified from various organisms, playing a main role in the browning of fruits and vegetables (enzymatic browning). Because browning is responsible for the loss of food quality, the inhibition of enzyme activity is an important topic in food science. The enzymes oxidize polyphenols to quinines, which are further polymerized nonenzymatically to melanins. Plant PPOs are nuclear-encoded proteins and synthesized as precursors. A plant precursor PPO contains two-domain transit peptides in its N-terminal region, which are signals for transportation to plastids and thylakoids.[5] The precursor PPO is routed to the lumen in two steps by plastids from plants. Importation to the stroma is followed by removal of the transit peptide in the N-terminal domain and the processed form accumulates in thylakoid lumens as the mature protein. Fungal PPOs have no signature for transit peptide and therefore are likely to be cytosolic enzymes.[6] The enzymes may be involved in sporulation, in the virulence of fungal pathogen, and in the defense of organs after injury.[7] Until the late 1980s, plant PPOs were thought to be 40-kDa proteins. In chloroplasts, the enzymes are 60 kDa, as judged by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE). Chloroplast enzymes are easily degraded in vitro and this degradation results in activation of the enzymes. Protease activity is thought to originate from PPO itself.

The cold-water extract of *A. brasiliensis* also shows pharmacological effects. The sequential browning of the extract suggests the existence of polyphenols that will be polymerized by polyphenoloxidases. We have demonstrated that the polyphenols produced by polyphenoloxidases from *Bupleuri radix*, a type of Chinese herbal medicine called Saiko in Japanese, have biological response-modifying effects.[8] This finding prompted us to speculate that polyphenols contained in the cold extract from *A. brasiliensis* are responsible for the biological activities. In mushrooms, PPO must be involved in the production of polyphenols and therefore we report here on the purification and characterization of PPO from *A. brasiliensis*.

II. MATERIALS AND METHODS

A. Mushroom Material and Reagents

Dry fruiting bodies of *A. brasiliensis* were imported from Brazil by Toei Pharmaceutical Co., Ltd. (Tokyo, Japan). We purchased 4-t-butylpyrocatechol (TBC), ammonium sulfate, SDS, L-cystein, ascorbic acid, 4-hydroxy anisol (4-HA, *p*-methoxyphenol), tropolone, and Silver stain kit II WAKO from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-3,4- Dihydroxyphenylalanine (L-DOPA) and Triton X-114 (TX-114) were purchased from Sigma (Tokyo, Japan). *p*-Coumaric acid and transferulic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Mimosine was obtained from ACROS (Geel, Belgium). The reagents used were of analytical grade.

B. Enzyme Purification

The enzyme was partially purified using TX-114 to remove phenols.[9–11] Dry powder of *A. brasiliensis* fruiting body was suspended in 0.1 M sodium phosphate buffer (pH 7.0) with 14.2 mM ascorbic acid and extracted at 4°C for 2 h. The extract was then centrifuged at 8000 x *g* for 20 min. The supernatant was subjected to TX-114 phase partitioning by adding twice the volume of 20% TX-114 (final detergent concentration was 13.3%), and the mixture was kept at 4°C for 10 min and then incubated at 37°C for 30 min. The resulting turbid solution was centrifuged at 8000 x *g* for 45 min and the upper phase was recovered. Ammonium sulfate was added to the detergent-poor solution to a final concentration of 40% saturation and stirred at 0°C for 1 h. After centrifugation at 8000 x *g* for 20 min at 4°C, ammonium sulfate was further added to the supernatant to a final concentration of 60% saturation and then incubated at 4°C overnight. The precipitate was collected by centrifugation at 8000 x

TABLE 1. Purification of *Agaricus brasiliensis* PPO

	Volume, mL	Total protein, mg	Total activity, units	Specific activity, units/mg	Purification, -fold	Recovery, %
Crude extract	15.5	599.8	898.4	1.5	1.0	100.0
Supernatant 13% TX-114	16.0	111.4	724.0	6.5	4.3	80.6
40–60% $(NH_4)_2SO_4$	0.8	6.4	283.5	44.3	29.6	31.6
Dialysis	1.6	5.4	274.7	50.9	34.0	30.6

g for 30 min and then resolved in 10 mM sodium phosphate buffer (pH 7.0). The salt was removed by dialysis and the enzyme solution was stored at −20°C.

C. Enzyme Activity

Diphenolase activity was determined spectrophotometrically using L-DOPA and *tert*-butylcathechol (TBC) as the substrates by recording dopachrome production at 492 nm ($\varepsilon = 3506.1\ M^{-1}\ cm^{-1}$) and tert-butylquinone production at 405 nm ($\varepsilon = 1136.2\ M^{-1}\ cm^{-1}$). One unit of polyphenoloxidase was defined as the amount of enzyme that produces 1 μmol quinone per minute. Monophenolase activity was measured spectrophotometrically using 4-hydroxyanisol (4-HA) as the substrate. Spectrophotometric measurements were performed with a Corona Microplate Reader MTP-450. The standard reaction medium contained 10 mM sodium phosphate buffer (pH 7.0), 1 mM SDS, and 1 mM or 5 mM TBC. The absorbance at 405 nm of each sample was measured in triplicate 5 min after initiation of the reaction.

D. Protein Concentration Determination

Protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard.

E. Partially Denaturing SDS-PAGE

Partially denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the method of Laemmli[10] with some modifications. Samples were processed in the absence of dithiothreitol (DTT) and without heating to preserve enzymatic activity, and applied to 11% polyacrylamide gel. After electrophoresis, the gel was stained for PPO activity in 10 mM sodium phosphate buffer (pH 7.0) containing 5 mM L-DOPA.[11,12] Kaleidoscope Prestained Standards (Bio-Rad, Hercules, CA, USA) was used as protein mass marker.

F. Kinetic Data Analysis

The Michaelis constant, K_m, and the maximum velocity, V_{max}, were determined from triplicate measurements in steady-state experiments. A nonlinear least-squares fitting program found in SigmaPlot (Systat Software, Richmond, UK) was used to estimate the apparent V_{max}, K_m and K_i values.

III. RESULTS

A. Partial Purification of Protein

Table 1 shows the summary of purification from *A. brasiliensis* fruiting bodies. In our early study, PPO was extracted from the powder of dry *A. brasiliensis* fruiting body with sodium phosphate buffer (pH 7.0) but the extract color sequentially became black. Accordingly, the extraction was carried out by adding ascorbic acid and aqueous two-phase partitioning to avoid browning. The partially purified enzyme was stored in phosphate buffer (pH 7.0) at 4°C until used for various experiments because the extract showed the PPO activity for several days.

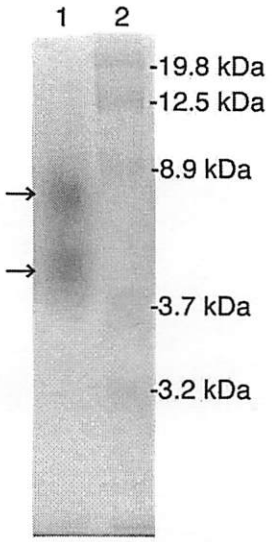

FIGURE 1. Partially denaturing SDS-PAGE (11% gel) of *Agaricus brasiliensis* PPO stained with 5 mM L-DOPA (lane 1) in 10 mM sodium phosphate buffer (pH 7.0). Lane 1, purified PPO stained with L-DOPA; lane 2, protein mass marker.

Partially denaturing SDS-PAGE was performed to identify the size of proteins showing PPO activity without DTT and heating to preserve enzymatic activity. As shown in Figure 1, two bands indicating PPO activity were observed and were approximately 70 kDa and 45 kDa. Because PAGE was not perfectly denatured, the protein size is probably not accurate.

B. Effect of Temperature

Purified PPO, dissolved in 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM SDS, was incubated at each temperature for 15 min, and then immediately chilled on ice and centrifugated. The supernatant was used to measure the remaining activity. The reaction substrate was 5 mM TBC. As shown in Figure 2, PPO activity was slightly increased at 40°C but clearly decreased at 60°C, which suggests that PPO protein may be denatured after heat treatment.

C. Activation of PPO by SDS

The effect of SDS on *A. brasiliensis* PPO activity was investigated (Fig. 3). The reaction medium included 1 mM TBC, purified PPO, and 10 mM sodium phosphate buffer (pH 7.0) added to a final concentration of 0.1–10 mM SDS, and then the absorbance of the reaction solution was measured with a microplate reader after 10 min. As shown in Figure 3A, PPO activity increased with increasing SDS concentration and several peaks were observed between 0.1–1 mM. This suggests that the activation mechanism of PPO accompanying conformational change of the active site occurred in several steps. The maximal activation was shown at 1.6 mM. A further increase in SDS concentration gave rise to a decrease in activity. The time-dependent change of diphenolase activity was observed as a sigmoid curve (Fig. 4).

D. Effect of pH

The pH profiles of *A. brasiliensis* PPO activity are dependent on the substrate used and in the absence or presence of SDS. In the case of TBC, maximal PPO activity was observed between pH 6–7 and PPO was activated in a higher range of pH by adding SDS; however, it was not activated in a low range of pH with SDS, whereas the latent form without SDS showed little activity at the optimum pH for the SDS-activated isoform (Fig. 5A). This suggests that PPO activity is affected by the components of buffers, and PPO from *A. brasiliensis*, especially applied at the active site by SDS, may

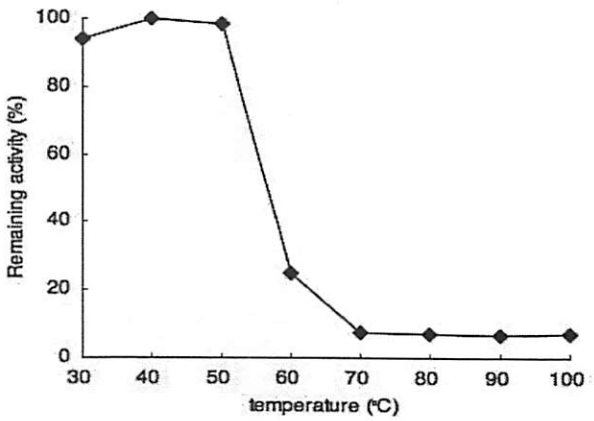

FIGURE 2. Remaining activity of *Agaricus brasiliensis* PPO at various temperatures. The reaction medium contained 10 mM phosphate buffer (pH 7.0), 1 mM SDS, and 5 mM TBC. Spectrophotometric measurements were performed 10 min after the reaction. Spectrophotometric measurements were performed 5 min after the reaction.

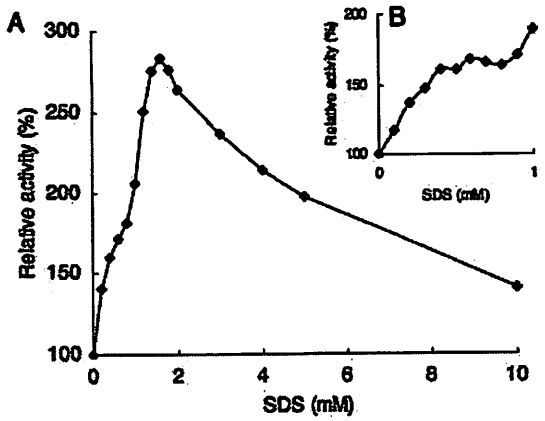

FIGURE 3. Effect of SDS concentration on *Agaricus brasiliensis* PPO activity. The reaction medium contained 10 mM phosphate buffer (pH 7.0) and 1 mM TBC. Spectrophotometric measurements were performed 10 min after the reaction. Native PPO activity is considered to be 100%. (**A**: SDS concentration, 0–10 mM; **B**: SDS concentration 0–1 mM)

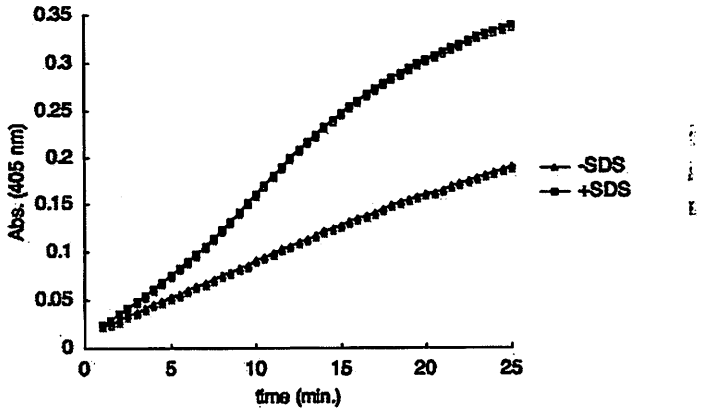

FIGURE 4. Time course of PPO reaction in the presence (1 mM, squares) or absence (triangles) of SDS. The reaction medium contained 10 mM phosphate buffer (pH 7.0) and 1 mM TBC. Spectrophotometric measurements were performed between 1–25 min after reaction.

be easily prevented by acetate buffer. In the case of L-DOPA, absorbance increased unusually in the middle of the reaction in the range of pH 7.5–8.0 (Fig. 5B), possibly because of the turbidity of the reaction solution; therefore, the maximal activity could not clearly observed.

E. Monophenolase and Diphenolase Activity of *A. brasiliensis* PPO

PPO activity with or without SDS was assayed at pH 7.0 using 4-hydroxyanisol (4-HA) as a substrate for monophenolase and L-DOPA as a substrate for diphenolase (Fig. 6). Activity was observed immediately in the reaction using L-DOPA and activated by SDS, whereas in the early stage of monophenolase reaction using 4-HA, the reaction did not occur with or without SDS, and a lag time was observed, but was shortened.

F. Inhibition of PPO Activity

The effect of inhibitors on PPO activity of *A. brasiliensis* was also analyzed (Table 2). The reaction medium included each concentration of inhibitors, which are general inhibitors of PPO or tyrosinase activity, and 1 mM TBC as the substrate, in the presence or absence of 1 mM SDS. In this experiment, we used the same reaction medium without enzyme as a control. Each sample was measured

TABLE 2. Percentage Inhibition of Purified *Agaricus brasiliensis* PPO by Reducing Agents and Substrate Analogues

	−SDS				1 mM SDS			
	1 μM	10 μM	100 μM	1 mM	1 μM	10 μM	100 μM	1 mM
Reducing agents								
Ascorbic acid	−1	13	69	105	3	16	68	99
L-cystein	3	8	78	99	1	3	37	100
Substrate analogues								
Mimosine	1	5	8	22	4	2	4	7
Tropolone	1	1	48	99	0	3	28	100
Cinnamic acid derivatives								
p-coumaric acid	18	19	21	−8	22	14	15	14
trans-ferulic acid	-3	1	1	−1	6	6	6	6

at 10 min. Although the reducing agents and analogue inhibitors inhibited PPO activity, cinnamic acid derivatives did not.

G. Kinetic Studies

The apparent kinetic parameters were calculated using the data obtained at pH 7.0 in the presence or absence of SDS. The apparent V_{max} value increased two-fold with SDS, but the K_m value hardly changed (Table 3 and Figure 8). In addition, we carried out similar experiments in the presence or absence of SDS using mimosine as an inhibitor to investigate the inhibition of diphenolase activity. The K_m and V_{max} values were not changed by mimosine. The K_i value of mimosine was increased by SDS (Table 3).

IV. DISCUSSION

It is difficult to purify enzymes from fungi because of the existence of various fungal compounds and the influence of their characteristics.[12] In our study, the extracts of *A. brasiliensis* fruiting bodies with phosphate buffer sequentially became black during purification. PPO in the extract polymerizes to phenol compounds and the color of sample solution affects the spectrophotometric measurement. We tried the protein purification method using an aqueous two-phase system at physiological temperature (at 22°C) based on Triton X-114 (TX-114), which was reported by Garcia-Carmona et al.[13-15] As a result, undesirable PPO activity in the extract could be suppressed by adding ascorbic acid as an antioxidant reagent, and the removal of phenols, as substrates, by TX-114, preventing browning of the enzyme solution as much as possible (Table 1).

In the partially denatured SDS-PAGE experiment, a smaller band stained with L-DOPA was observed in addition to the desirable band (Fig. 1). After translation, the C-terminal peptide of *Neurospora crassa* tyrosinase was cleaved, resulting in 46 kDa of mature enzyme.[6,16] The Phe_{407} of protyrosinase from *N. crassa* is the chymotryptic cleavage site and Tyr_{381} in PPO cDNA of *A. brasiliensis* is the corresponding site,[17,18] which suggests that PPO of *A. brasiliensis* is in the same group as tyrosinase of *N. crassa*.

The effect of temperature on purified PPO was

investigated (Fig. 2). The remaining activity slightly increased at 40°C but decreased at 60°C. The fruiting bodies of *A. brasiliensis* as raw material were dried at 60°C for product commercialization and disagreed with these results; however, there are many components in the fruiting body that are reciprocally related and the extracts contain minor thermostable components. As a result, the enzyme may maintain activity slightly between 70°C and 100°C.

The effect of pH is an important factor for the exertion of enzyme activity.[19] In this study, the optimal pH value could not be determined (Fig. 5) because of the turbidity of the reaction solution. It is suggested that the pH profiles of *A. brasiliensis* PPO activity were dependent on several factors.

Polyphenoloxidase (tyrosinase) exists as two forms: latent (inactive) and active.[6,7] Enzymes in latent form are activated by several factors, such as alcohols, acid treatment, proteases, and anionic detergent (e.g., SDS), through conformation change.[20] SDS inactivates most enzymes but activation of latent enzyme by SDS is a common feature of other latent PPOs and was observed in *A. brasiliensis* PPO (Fig. 3). Espin and Wichers[21] indicated that the protease-treated enzyme showed a smaller band in SDS-PAGE but the SDS-activated enzyme showed an optimum pH different from that of the protease-activated isoform. As compared to this report, it was suggested that PPO from *A. brasiliensis* was similar to the SDS-activated isoform and the lower band of SDS-PAGE was not protease-activated PPO.

PPO catalyses two distinct reactions: monophenolase (hydroxylation) and diphenolase (oxidation) activity. These steps lead to the formation

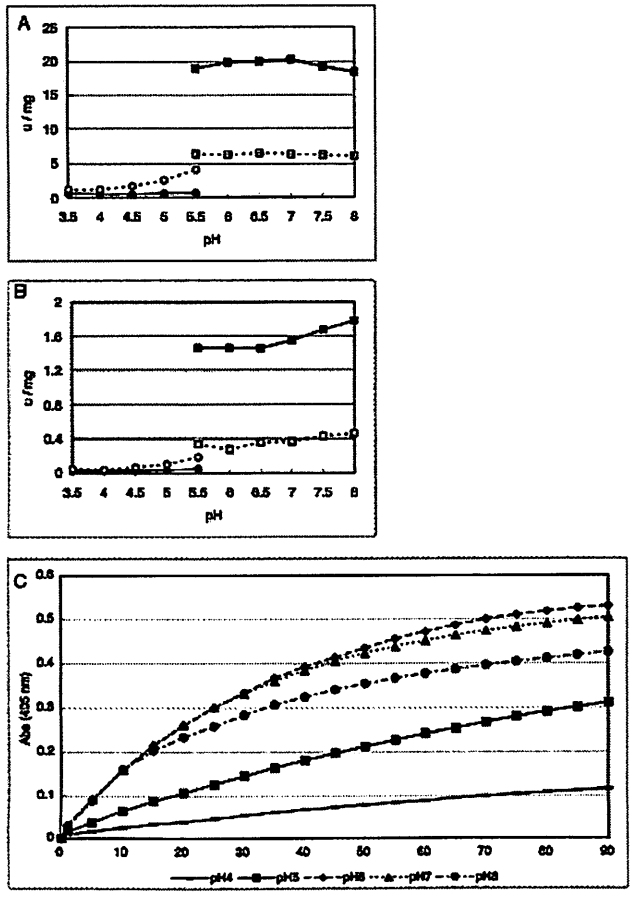

FIGURE 5. Effect of pH on the activity of *Agaricus brasiliensis* PPO in 10 mM sodium acetate (pH 3.5–5.5, circles) and 10 mM sodium phosphate (pH 5.5–8.0, squares) in the presence (solid symbols) or absence (open symbols) of 1 mM SDS using TBC (**A**), (**C**) or L-DOPA, (**B**) as a substrate.

TABLE 3. Kinetic Parameters of Native and SDS Activated Purified PPO

	− Mimosine		+ Mimosine	
	−SDS	+SDS	−SDS	+SDS
K_m (TBC; mM)	0.56 ± 0.03	0.47 ± 0.10	0.42 ± 0.04	0.56 ± 0.04
apparent V_{max} (units/mg total protein)	17.4 ± 0.34	38.8 ± 2.46	13.8 ± 0.32	33.9 ± 0.62
$K_{I\,(mimosine)}$ (x 10^{-6} M)	ND	ND	138.2 ± 19.5	281.0 ± 31.0

Abbreviation: ND, not determined.

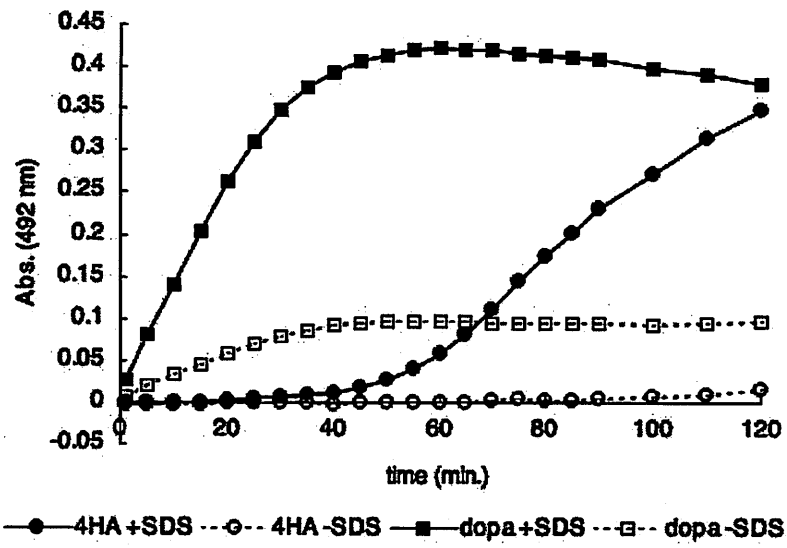

FIGURE 6. Enzyme activity of *Agaricus brasiliensis* PPO in the presence (1 mM, solid symbols) or absence (open symbols) of SDS. Reaction medium contained 1 mM L-DOPA (squares) or 4-HA (circles) in 10 mM phosphate buffer (pH 7.0).

of *o*-quinones and follow nonenzymatic steps using molecular oxygen, resulting in the formation of melanin. Monophenolase activity relates to lag time, which is observed by hydroxylation and the reaction curves sigmoidally (Fig. 6). The lag time is dependent on the characteristics of enzymes, concentration, substrate, existence of impurities, temperature, and pH, and disappears or shortens with an aliquot of reductant, especially *o*-diphenols.

The PPO has an active site for both substrates, containing two Cu binding motifs: CuA (N-terminal side) and CuB (C-terminal side).[6,22] The three types of binuclear Cu sites are *Met*-form (inactive and stable), *Oxy*-form (active and unstable), and *Deoxy*-form (not binding oxygen). The oxygen molecules exist as peroxide in the *Oxy*-form. Monophenol can be oxidized with the *Oxy*-form of the enzymes, whereas the *Met*-form cannot react from monophenols to diquinones but leads to the "dead-end pathway." The lag time of monophenolase activity reflects the reaction mechanism of autocatalysis to produce diphenols in the early stage of the monophenolase reaction. Whereas the lag time does not occur in diphenolase activity, because *o*-diphenol can be oxidized to *o*-quinone with both the *Met*-form and *Oxy*-form, and absorption begins to increase immediately (Fig. 6). Figure 4 suggests that diphenols react only with the *Met*-form and that this time may reflect the early stage of the sigmoid curve.

Inhibition by reducing agents (thiol com-

pounds) is an additional reaction that takes place with quinones to form stable colorless products and/or to bind to the active center of the enzyme. Ascorbic acid acts as an antioxidant, rather than an inhibitor, because it reduces the initial quinone formed by the enzyme to the original diphenol before it undergoes the secondary reactions that lead to browning.[10,23] On the other hand, tropolone and mimosine act as both analogue inhibitors and chelation reagents,[7,24] and are suggested to be competitive inhibitors with other polyphenoloxidases or tyrosinases. The *Oxy*-form is an obligatory intermediate in diphenolase catalytic turnover and the presence of the substrate is necessary for the action mechanism of slow binding inhibitors.[7,12] As shown in Figure 7, inhibition by tropolone was characterized by the lag time using *A. brasiliensis* PPO as an enzyme. As mentioned above, tropolone inhibits the slight amount of the *Oxy*-form in the earlier reaction medium and thus only the *Met*-form can product *o*-diquinone. In other words, the lag time shows that only the *Met*-form has reacted.

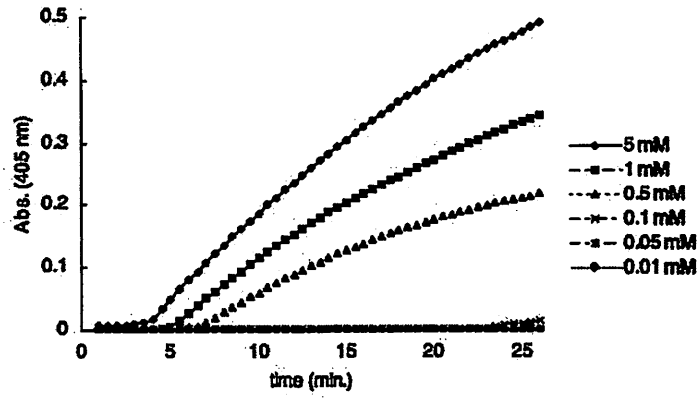

FIGURE 7. Inhibition of PPO activity by tropolone. The reaction medium contained 10 mM sodium phosphate buffer (pH 7.0), 1 mM SDS, and 0.01–5 mM TBC using 300 μM tropolone as an inhibitor. Spectrophotometric measurements were performed at 405 nm between 1–26 min after the reaction.

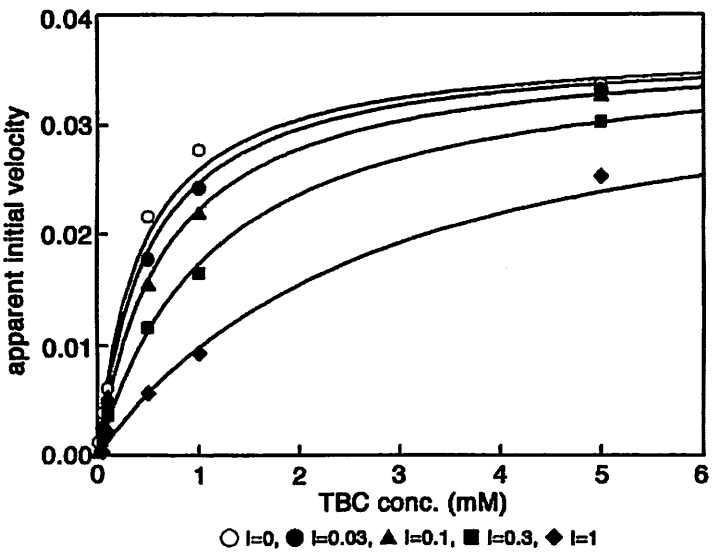

FIGURE 8. Kinetic study of PPO inhibition. The reaction medium contained 10 mM sodium phosphate buffer (pH 7.0), 1 mM SDS, and 0.01–5 mM TBC using mimosine (30–1000 μM) as an inhibitor. Spectrophotometric measurements were performed at 405 nm between 1–26 min after reaction.

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