Inhibition of Mushroom Polyphenol Oxidase by Agaritine

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Agaritine [(*β*-*N*-(*γ*-L(+)-glutamyl)-4-hydroxymethylphenylhydrazine] was isolated and purified from mushrooms (*Agaricus bisporus*). This paper reports for the first time the inhibition of the monophenolase and diphenolase activities of mushroom polyphenol oxidase by this abundant and characteristic compound from the *Agaricus* genus. Agaritine showed uncompetitive inhibition (K_I = 2.3 mM) and partial competitive inhibition (K_I = 0.13 mM; α = 2.5) when L-DOPA and L-tyrosine were assayed, respectively. In addition to the ability of agaritine to inhibit the enzyme per se, this compound was capable of removing the enzymatically generated *o*-quinones in the assay. To solve this problem, the inhibition was characterized by using 3-methyl-2-benzothiazolinone hydrazone as nucleophile reagent to form a stable nucleophile−quinone adduct that could not be attacked by agaritine. The inhibition of mushroom polyphenol oxidase by other inhibitors is compared to the inhibition by agaritine. Moreover, a possible role for agaritine in the browning of mushrooms is discussed.

Keywords: *Agaritine; depigmentation; inhibition; MBTH; mushroom; polyphenol oxidase*

INTRODUCTION

Polyphenol oxidase (EC 1.14.18.1; PPO) is the main enzyme involved in the enzymatic browning of mushrooms and other crops. The darkening of food products, although innocuous to consumers, causes a decrease in market value and economic loss because it connotes spoilage. PPO catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) which, in turn, are polymerized to brown, red, or black pigments (Prota, 1988; Sánchez-Ferrer et al., 1995). The prevention of this browning reaction has always been a challenge to food scientists (Kahn and Andrawis, 1984; Janovitz-Klapp et al., 1990; Chen et al., 1991a,b).

Agaricus species are known for their ability to synthesize *γ*-glutamyl aromatic amino acids derived from *para*-C1-substituted phenylhydrazines such as agaritine [(*β*-*N*-(*γ*-L(+)-glutamyl)-4-hydroxymethylphenylhydrazine] (Levenberg, 1962; Soulier et al., 1993). In previous reports, agaritine content in fresh mushrooms was found to be in the range of 94−629 mg/kg fresh weight and 2.1−6.9 g/kg in dried commercial mushrooms (Fisher et al., 1984). The fructifying mycelium appeared to be a site of intense synthesis of agaritine, which also was found throughout the sporophore with lowest levels in stipe base and highest in lamellae (Soulier et al., 1993).

Agaritine is a good substrate for purified *γ*-glutamyl transpeptidase being converted to a major metabolite, 4-(hydroxymethyl)phenylhydrazine, formed as a result of the loss of the glutamyl moiety (Walton et al., 1997). Agaritine deserves particular attention as precursor of potentially toxic aryldiazonium ions although contradictory reports on the possible contribution of agaritine in the mutagenicity of edible mushroom *Agaricus bisporus* have been reported (Paparaskeva-Petrides et al., 1993; Walton et al., 1997). This molecule is of fundamental interest because of the central position of the glutamyl residue in fungal nitrogen metabolism (Moore, 1984). However, to the best of our knowledge, this is the first report of the effect of agaritine on mushroom polyphenol oxidase activity.

Other fungal metabolites such as kojic acid [5-hydroxy-2-(hydroxymethyl)-*γ*-pyrone] are known inhibitors of mushroom polyphenol oxidase (Chen et al., 1991a,b; Kahn et al., 1995). Moreover, this compound was able to reduce *o*-quinones to diphenols to prevent the final pigment (melanin) forming (Chen et al., 1991b; Kahn et al., 1995).

The aim of this paper is to describe the inhibition of the monophenolase and diphenolase activities of mushroom polyphenol oxidase by agaritine. This effect has not been previously reported in the literature. In addition, these findings call for caution in the use of agaritine in experiments with *o*-quinone accumulation due to the depigmenting effect of agaritine. Moreover, a possible role for agaritine in the browning of mushrooms is discussed.

MATERIALS AND METHODS

Reagents. L-DOPA, L-tyrosine, 4-*tert*-butylcatechol (*t*BC), and MBTH were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade and supplied by Merck (Darmstadt, Germany). Stock solutions of phenolic compounds were prepared in 0.15 mM *o*-phosphoric acid as a solvent to prevent autoxidation. Milli-Q system (Millipore Corp., U.S.A.) ultrapure water was used throughout this research. To dissolve the MBTH−quinone adducts, 2% (v/v) *N*,*N*′-dimethylformamide (DMF) was added to the assay medium (Winder and Harris, 1991).

Enzyme Source. Mushroom (*Agaricus bisporus*) polyphenol oxidase (3900 units/mg) was purchased from Sigma. The commercial preparation of PPO showed a single isoenzyme

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with a isoelectric point around 4.1 determined by isoelectric focusing and a major band of M_r of 43 000 in SDS−PAGE electrophoresis (results not shown). There was no contamination of laccases or peroxidases in the commercial preparation because when tropolone (a specific inhibitor of PPO) was added to the assay medium, in routine experiments, no activity could be measured.

Isolation and Purification of Agaritine. Agaritine was extracted from the gills of fresh *A. bisporus* fruit bodies U1 stage 3. One hundred milligrams of gills was ground three times in a blender with 200 mL of 0.5% (w/v) sodium bisulfite in 1% acetic acid. Lysates were filtered and centrifuged for 20 min at 10000*g*. Supernatants were pooled, and the crude extract was taken to dryness; dry material was then dissolved into 5 mL of distilled water and applied to the chromatographic column. The ion-exchange resins were prepared as described by Redgwell (1980). The anion exchanger QAE Sephadex A-25 was activated 2 days in 0.5 M sodium formate and after filtration was stored in 0.05 M sodium formate. The anion exchange column (length 80 cm, diameter 2.5 cm) was eluted with water (1 mL/min), allowing a coarse separation of agaritine. Fractions containing the phenylhydrazine were pooled and taken to dryness. Dry residue was suspended in distilled water and injected on an HPLC column Lichrosorb (length 25 cm, diameter 2.5 cm, particle size 100 Å), RP C18 eluted with MeOH 10% at a flow rate of 2 mL/min. Fractions containing agaritine were pooled and taken to dryness. Agaritine was crystallized in hot ethanol. Purity was monitored by HPLC using a Nucleosil (length 25 cm, diameter 0.5 cm, particle size 100 Å) C 18 column eluted with acetic acid/MeOH/water (4:20:976) at a flow rate of 0.8 mL/min.

Enzymatic Assays. The use of MBTH as a nucleophilic reagent on some *o*-quinones generated by PPO has been previously described for the measurement of diphenolase and monophenolase activities of PPO from several sources (Espín et al., 1995a,b; 1996; 1997a−e). The diphenolase and monophenolase activities of mushroom PPO were assayed spectrophotometrically at 484 nm using L-DOPA and L-tyrosine as substrates, respectively.

The spectrophotometric assays were recorded in an ultraviolet−visible Perkin-Elmer Lambda-2 spectrophotometer (Überlingen, Germany), on-line interfaced to a 486-DX33 microcomputer (Ede, The Netherlands). Temperature was controlled at 25 °C with a circulating bath with heater/cooler and checked using a precision of ±0.1 °C. The reference cuvette contained all the components except the substrate with a final volume of 1 mL.

Kinetic Data Analysis. The values of K_m and V_m were calculated from triplicate measurements of the steady-state rate, V_{ss}, for each initial substrate concentration ($[S]_0$). The reciprocal of the variances of V_{ss} were used as weighting factors to the nonlinear regression fitting of V_{ss} vs $[S]_0$ to the Michaelis equation (Wilkinson, 1961; Endrenyi, 1981). The fitting was carried out by using a Gauss−Newton algorithm (Marquardt, 1963) implemented in the Sigma Plot 2.01 program for Windows (Jandel Scientific, 1994).

RESULTS AND DISCUSSION

Formation of MBTH−Quinone Adducts. Spectrophotometric recordings measuring *o*-quinone accumulation enzymatically generated from the oxidation of several *o*-diphenols yielded nonreliable recordings because of the depigmenting effect of agaritine (Figure 1). This effect was previously reported for kojic acid (Chen et al., 1991b; Kahn et al., 1995). Therefore, experiments measuring the accumulation of a highly stable *o*-quinone such as 4-(*tert*-butyl)benzo-1,2-quinone (*t*BQ) (Ros et al., 1994a) could render erroneous results because of the depigmenting artifact. For example, in determined assay conditions, the spectrophotometric recordings obtained in the presence of agaritine could suggest a time-dependent inhibition by a slow-binding

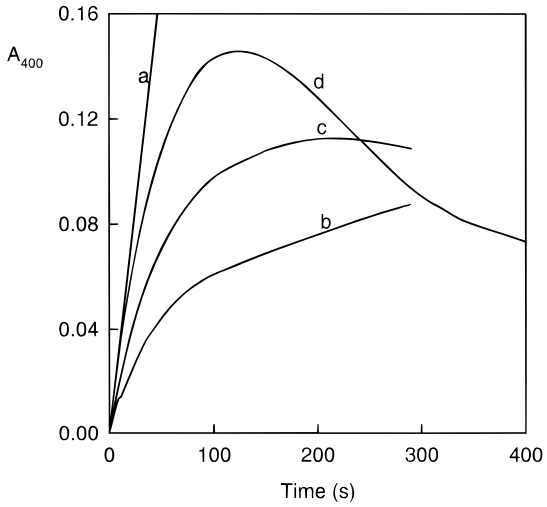

Figure 1. Spectrophotometric recordings of *o*-quinone accumulation for the diphenolase activity of mushroom polyphenol oxidase. The reaction medium included 50 mM PB, pH 6.8, 22 nkat/mL mushroom polyphenol oxidase and (a) 3 mM *t*BC; (b) 0.4 mM *t*BC and 3 mM agaritine; (c) 1 mM *t*BC and 3 mM agaritine; and (d) 3 mM *t*BC and 3 mM agaritine.

inhibitor with a decrease in the initial velocity to a steady-state inhibited velocity (Figure 1, curve b). However, by changing the assay conditions (Figure 1, curves c and d), a decrease in the absorbance could be observed. Taking into account that *t*BQ was very stable in the pH of the assay medium, the shape of the spectrophotometric recordings could be explained by the presence of two overlapping reactions (enzymatic generation of *o*-quinone and disappearance of *o*-quinone by the attack of agaritine). Depending on the assay conditions, the formation or decomposition reaction prevailed. To solve this problem, MBTH was used as a coupled nucleophile to form the MBTH−quinone adduct (Espín et al., 1995a,b, 1996, 1997a−e). Since *t*BQ was a highly stable *o*-quinone (Ros et al., 1994a) that did not react with MBTH, then L-DOPA and L-tyrosine, also abundant metabolites in mushrooms, were used as substrates for determining the inhibition of the diphenolase and monophenolase activities of mushroom PPO by agaritine, respectively.

The spectrum of MBTH−quinone adduct was the same in the absence and in the presence of agaritine (results not shown). Therefore, the MBTH assay method was used to study the net effect of the inhibition of mushroom PPO by agaritine without artifacts due to the depigmenting effect of agaritine.

Although this work has been mainly focused on the spectrophotometric study of the inhibition of mushroom polyphenol oxidase by agaritine, the oxygen consumption in both monophenolase and diphenolase activities was also recorded (results not shown). A decrease in the O_2 consumption could be observed in the presence of agaritine.

Inhibition of Mushroom Polyphenol Oxidase by Agaritine. Agaritine showed a simple linear uncompetitive inhibition when L-DOPA was used as a substrate. The dependencies obtained (Figures 2 and 3) justified this type of inhibition (Segel, 1976). Agaritine decreased V_m and K_m values to the same extent (Table 1). An inhibition constant (K_I) value of 2.3 mM was obtained at the optimum pH of mushroom PPO. The inhibition power of several commercially available inhibitors was compared in the diphenolase activity of

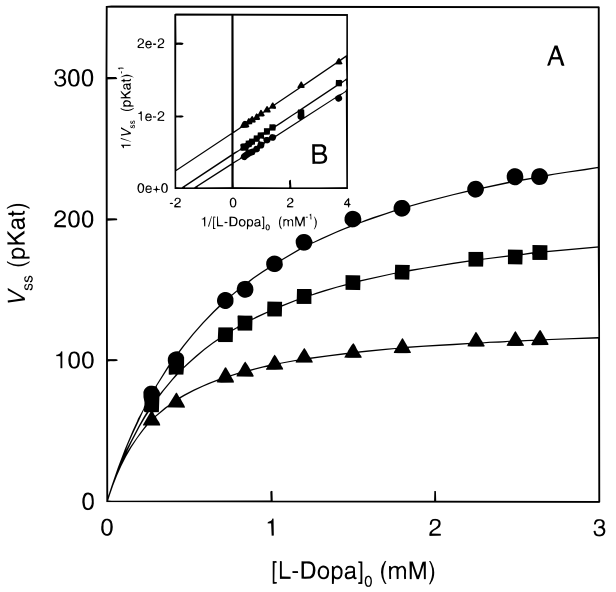

Figure 2. (A) Dependence of V_{ss} on initial L-DOPA concentration. (B) Lineweaver−Burk plot. The reaction medium included 50 mM PB, pH 6.8, 2% DMF, 5 mM MBTH, 22 nkat/mL mushroom polyphenol oxidase, (0.17−2.7) mM L-DOPA, and (●) 0 mM agaritine; (■) 1 mM agaritine; and (▲) 4 mM agaritine.

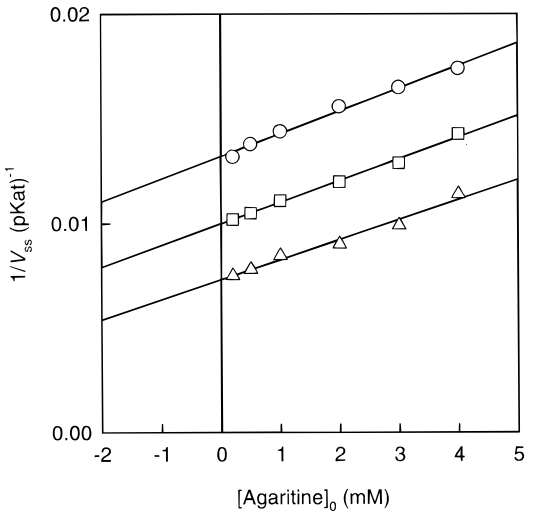

Figure 3. Dependence of $1/V_{ss}$ on initial agaritine concentration (Dixon plot). The reaction medium included 50 mM PB, pH 6.8, 2% DMF, 5 mM MBTH, 22 nkat/mL mushroom polyphenol oxidase, (0.2−4) mM agaritine and (○) 0.28 mM L-DOPA; (□) 0.42 mM L-DOPA and (△) 0.7 mM L-DOPA.

Table 1. V_m and K_m Values for the Diphenolase and Monophenolase Activities of Mushroom Polyphenol Oxidase in the Presence of Agaritine[a]

	L-DOPA		L-tyrosine	
[agaritine]$_0$ (mM)	V_m (pkat)	K_m (mM)	V_m (pkat)	K_m (mM)
0	300 ± 20	0.81 ± 0.06	6.6 ± 0.3	0.26 ± 0.02
0.5			6.3 ± 0.3	0.37 ± 0.02
1	210 ± 14	0.56 ± 0.03	6.8 ± 0.3	0.63 ± 0.04
4	130 ± 9	0.35 ± 0.02		

[a] Conditions were 2% DMF, PB 50 mM, pH 6.8, 5 mM MBTH, and mushroom polyphenol oxidase 22 nkat/mL.

mushroom PPO (Table 2). The most potent inhibitor assayed for PPO activity was tropolone (Kahn and Andrawis, 1985). L-Mimosine (Cabanes et al., 1986; Kahn and Andrawis, 1985), benzoic acid (Menon et al., 1990), and agaritine caused approximately the same inhibition at the inhibitor concentrations assayed. The least inhibition was observed when azelaic acid was used (Shallreuter and Wood, 1990).

Table 2. Comparison of the Inhibition of Mushroom Polyphenol Oxidase with Several Inhibitors[a]

		% inhibition	
inhibitor	inhibitor concn (mM)	diphenolase activity	monophenolase activity
control	0	0	0
agaritine	0.2	21	20
	2	49	45
azelaic acid	0.2	3	1
	2	11	3
benzoic acid	0.2	30	25
	2	55	57
L-mimosine	0.2	28	25
	2	45	58
tropolone	0.2	95	99
	2	99	100

[a] Conditions were 2% DMF, PB 50 mM pH 6.8, 5 mM MBTH, mushroom polyphenol oxidase 22 nkat/mL, and 0.2 or 2 mM of the correponding inhibitor.

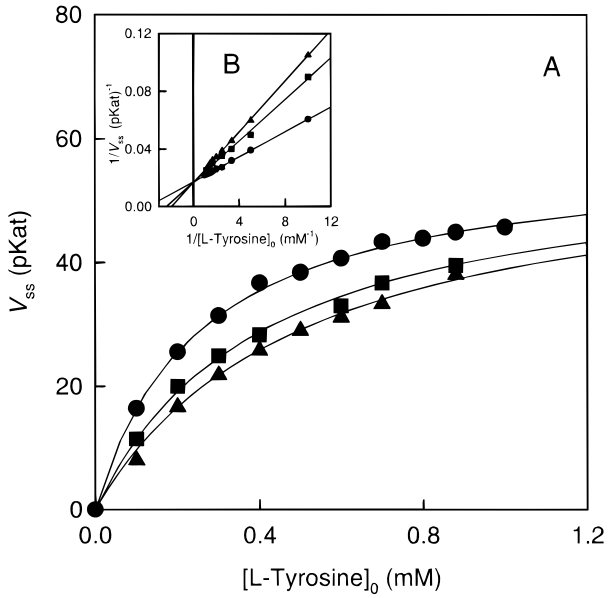

Figure 4. (A) Dependence of V_{ss} on initial L-tyrosine concentration. (B) Lineweaver−Burk plot. The reaction medium included 50 mM PB, pH 6.8, 2% DMF, 5 mM MBTH, 0.2 μkat/mL mushroom polyphenol oxidase, (0.1−0.9) L-tyrosine mM and (●) 0 mM agaritine; (■) 0.5 mM agaritine; and (▲) 1 mM agaritine.

Agaritine showed a simple intersecting hyperbolic competitive inhibition (partial competitive inhibition) when L-tyrosine was used as substrate. The dependencies obtained (Figures 4−6) justified this type of inhibition (Segel, 1976). Agaritine increased K_m values and did not change V_m values (Table 1). Partial competitive inhibition cannot be distinguished from pure competitive inhibition simply by plotting the steady-state rate (V_{ss}) versus substrate concentration ([S]) (Figure 4A) in the presence or absence of inhibitor or by the corresponding reciprocal plots (Figure 4B). It can be distinguished, however, by plotting V_{ss} versus [I] at a fixed [S] (Figure 5) or by the Dixon plot (Figure 6). The velocity of the reaction can never be driven to zero. The net effect of an infinitely high [I] is to produce a modified, but functional, enzyme (Figure 5). An inhibitor constant (K_I) value of 0.13 mM was obtained

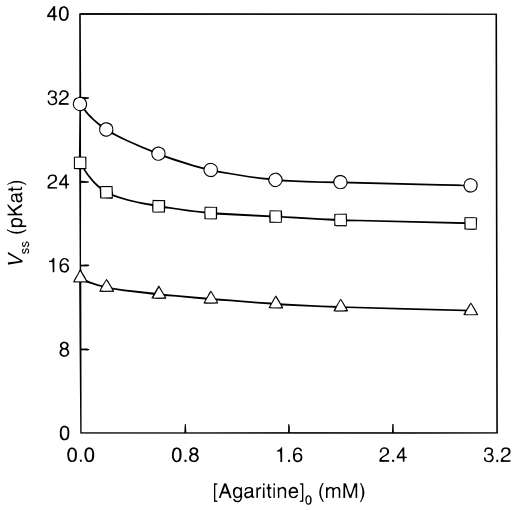

Figure 5. Dependence of V_{ss} on initial agaritine concentration. The reaction medium included 50 mM PB, pH 6.8, 2% DMF, 5 mM MBTH, 0.2 μkat/mL mushroom polyphenol oxidase, (0.2−2.8) mM agaritine and (△) 0.1 mM L-tyrosine; (□) 0.3 mM L-tyrosine; and (○) 0.5 mM L-tyrosine.

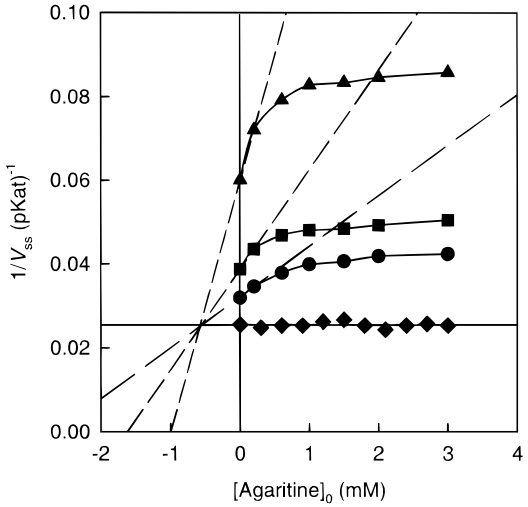

Figure 6. Dependence of $1/V_{ss}$ on initial agaritine concentration (Dixon plot). Conditions were the same as in Figure 5. Dashed lines show the deviation from linear competitive inhibition.

at the optimum pH of mushroom PPO. The factor α was 2.5 (Segel, 1976). This is the factor by which K_m changes when the inhibitor (I) binds the enzyme. K_I and α values were obtained by plotting the inverse of the slopes of Lineweaver−Burk plot (Figure 4B) versus the inverse of [I] (Segel, 1976) (results not shown). The partial competitive inhibition of mushroom polyphenol oxidase by agaritine in the presence of monophenol might be explained, in part, because of the similarity between the structures of agaritine and γ-glutamylhydroxybenzene, one of the most abundant monophenols in *A. bisporus* mushroom (Soulier, 1993).

The inhibition of the monophenolase activity of mushroom PPO was compared by using several known commercially available inhibitors (Table 2). The results of the comparison among the different inhibitors was the same as that previously observed for the diphenolase activity. The most potent inhibitor assayed for the monophenolase activity of mushroom PPO was tropolone (Kahn and Andrawis, 1985). V_{ss} could not be determined in 2 h of kinetic assay due the decrease of V_{ss} and to the increase of the lag period (Kahn and Andrawis, 1985). Agaritine showed approximately the same inhibition power as benzoic acid and L-mimosine. The lowest inhibition was achieved by azelaic acid, which almost did not inhibit the monophenolase activity at the inhibitor concentrations assayed (Schallreuter and Wood, 1990) (Table 2).

Type of inhibition (with the same inhibitor and enzyme) has been previously been reported to differ depending on the substrate assayed (Menon et al., 1990; Schallreuter and Wood, 1990; Chen et al., 1991a,b; Kermasha et al., 1993; Cabanes et al., 1994). The diphenolase and monophenolase activities of polyphenol oxidase involve one single (oxidase) and two overlapping (hydroxylase and oxidase) catalytic cycles, respectively. The stoichiometry of the pathway implies that one molecule of polyphenol oxidase must accomplish two turnovers in the hydroxylase cycle for each cycle in the oxidase activity. Polyphenol oxidase has only one active site to catalyze both activities (Rodríguez-López et al., 1992; Ros et al., 1994a,b; Solomon, 1996). This complex reaction mechanism, depending on the substrate used (*o*-diphenol or monophenol), renders complex equations for V_m and K_m involving many binding and rate constants (Rodríguez-López et al., 1992; Ros et al., 1994a,b). Maybe the complexity of this reaction mechanism could explain the different types of inhibition depending on the reaction mechanism that is taking place. Therefore, these results agree with those obtained for other inhibitors such as benzoic acid, azelaic acid, or kojic acid, which showed different types of inhibition on PPO depending on the substrate used (Menon et al., 1990; Schallreuter and Wood, 1990; Chen et al., 1991a,b; Kermasha et al., 1993; Cabanes et al., 1994).

CONCLUSIONS

The inhibition of mushroom polyphenol oxidase by agaritine has been reported for the first time. Moreover, agaritine showed a depigmenting effect preventing melanin formation. The inhibition was uncompetitive when L-DOPA was used as the substrate and partially competitive when L-tyrosine was used as the substrate. Taking into account that agaritine is very abundant in *A. bisporus* mushrooms, it might be suggested that agaritine could play in vivo a role as endogenous regulator of the mushroom polyphenol oxidase activity and of *o*-quinone concentration formed. The use of MBTH for the study of the inhibition of polyphenol oxidase by agaritine is strongly recommended because of the capability of agaritine for removing *o*-quinones from the assay medium. Moreover, these findings call for the synthesis of agaritine-related compounds to prevent the browning of fruits and vegetables.

ABBREVIATIONS USED

Agaritine, (*β*-*N*-(*γ*-L(+)-glutamyl)-4-hydroxymethylphenylhydrazine; DMF, *N*,*N*′-dimethylformamide; L-DOPA, L-3,4-dihydroxyphenyl alanine; I, inhibitor (agaritine); K_I, inhibition constant; K_m, Michaelis constant; M, monophenol; MBTH, 3-methyl-2-benzothiazolinone hydrazone; MeOH, methanol; PB, sodium phosphate buffer; PPO, polyphenol oxidase; S, substrate; SDS−PAGE, electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate; V_m, maximum steady-state rate; V_{ss} steady-state rate; *t*BC, 4-*tert*-butylcatechol; *t*BQ, 4-(*tert*-butyl)benzo-1,2-quinone.

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