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Agaritine from *Agaricus bisporus* is capable of preventing melanin formation

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Abstract

Agaritine [(β-*N*-(γ-L(+)-glutamyl)-4-hydroxymethylphenylhydrazine] was isolated and purified from the mushroom *Agaricus bisporus*. The prevention of melanin formation by agaritine is reported here for the first time. This compound, characteristic for the *Agaricus* genus, was a nucleophile in its deprotonated state which was able to remove the, either enzymatically or chemically generated, *o*-quinones from the assay medium. The disappearance of these *o*-quinones followed first order kinetics through a decreasing uniexponential. The dependencies of the apparent rate constant (k_q) for the disappearance of the *o*-quinone, as well as the *o*-quinone concentration removed from the assay medium ($[Q]_0$) were studied as a function of proton, agaritine and *o*-quinone initial concentrations. k_q increased linearly with agaritine concentration, decreased non-linearly with proton concentration and was independent of the initial *o*-quinone concentration. $[Q]_0$ showed a linear increase with initial *o*-quinone concentration, a non-linear decrease with proton concentration and was independent of the initial agaritine concentration. Taking into account the effect of agaritine on *o*-quinones, this paper calls for caution in the use of agaritine in experiments with *o*-quinone accumulation. Moreover, a possible role for agaritine in the browning of mushrooms is discussed.

Keywords: *Agaricus bisporus*; *Agaricaceae*; Kinetics; Agaritine; Melanin; Mushroom; *O*-quinone; Tyrosinase

1. Introduction

Agaricus species are known for their ability to synthesise typical γ-glutamyl aromatic amino acids such as agaritine (β-*N*-(γ-L(+)-glutamyl)-4-hydroxymethylphenylhydrazine (Levenberg, 1962; Soulier, Foret & Arpin, 1993). This molecule, occurring in *Agaricus bisporus*, is of fundamental interest because of the central position of the glutamyl residue in fungal nitrogen metabolism (Moore, 1984). It is also especially interesting because of its potential role in the synthesis of toxic aryldiazonium ions. However, contradictory results of studies on the possible contribution of agaritine to the mutagenicity of edible mushroom *A. bisporus* have been reported so far (Toth, Patil & Jae, 1984; Paparaskeva-Petrides, Ioannides & Walker, 1993; Walton, Coombs, Catterall, Walker & Ioannides, 1997). In previous reports, agaritine was found in the fruiting body mushroom in all tissues with lowest levels in stipe base and highest in lamellae (Soulier et al., 1993). Its content was found in a range 94–629 mg/kg of fresh material and 2.1–6.9 g/kg in dried commercial mushrooms (Fischer, Lüthy & Schlatter, 1984).

Tyrosinase (EC 1.14.18.1; PPO) is the main enzyme involved in the enzymatic browning of mushrooms which is responsible for sensory quality loss and loss of nutrient quality. This enzyme catalyses 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 polymerised to brown, red or black pigments (Sánchez-Ferrer, Rodríguez-López, García-Cánovas & García-Carmona, 1995; Prota, 1988). The prevention of this browning reaction has always been a challenge to food scientists (Kahn & Andrawis, 1984; Cabanes, García-Cánovas, Tudela, Lozano & García-Carmona, 1986; Janovitz-Klapp, Richard, Goupy & Nicolas, 1990; Chen, Wei, Rolle, Otwell,

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Balaban et al., 1991a). Previous studies on kojic acid [5-hydroxy-2-(hydroxymethyl)-γ-pyrone] (Kahn, Lindner & Zakin, 1995; Chen, Wei & Marshall, 1991b) and azide (Sugumaran, 1995) which are known inhibitors of mushroom tyrosinase reported the prevention of melanin formation in their presence. These compounds, in addition to the ability to inhibit the enzyme per se, are able to change the spectrum of some pigmented products, probably due to the ability of enzymatically *o*-quinones formed to oxidise kojic acid to a yellow product (in the presence of kojic acid) (Kahn et al., 1995; Chen et al., 1991b) and the formation of azido-catechol (in the presence of azide as inhibitor and catechol as substrate) (Sugumaran, 1995).

The aim of the work presented here was to study kinetically the effect of agaritine for removing the *o*-quinones formed either enzymatically or chemically. To this purpose, two parameters of the decreasing uniexponential, the apparent rate constant for the disappearance of *o*-quinone (k_q), and the *o*-quinone concentration removed in the assay ($[Q]_0$), are studied as a function of proton, *o*-quinone and agaritine concentrations. From these assays a possible role of agaritine in the prevention of melanin formation might be suggested.

2. Results and discussion

One of the most widely used assay methods to measure tyrosinase activity is the determination of the enzymatically-generated *o*-quinones (Duckworth & Coleman, 1970; García-Carmona, Pedreño, Galindo & García-Cánovas, 1979). This method has some disadvantages due to the instability of *o*-quinones and their further polymerisation to melanins. However, the diphenolase activity of tyrosinase can be followed by using 4-*tert*-butylcatechol (*t*BC) which yields the highly stable 4-(*t*-butyl)benzo-1,2-quinone (*t*BQ) (Ros, Rodríguez-López & García-Cánovas, 1994a).

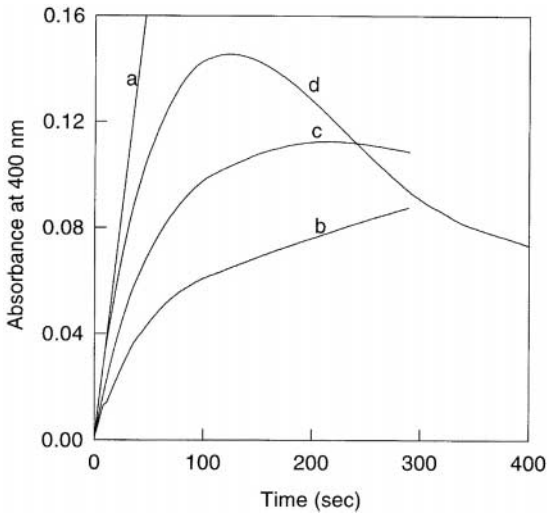


Fig. 1. Spectrophotometric recordings of *o*-quinone accumulation in the diphenolase activity of mushroom tyrosinase. The reaction medium contained 50 mM sodium phosphate buffer (PB) pH 6.8, 22 nkat/ml mushroom tyrosinase and, curve a: 3 mM *t*BC; curve b: 0.4 mM *t*BC and 1.5 mM AG; curve c: 1 mM *t*BC and 1.5 mM AG; curve d: 3 mM *t*BC and 1.5 mM AG.

The reaction mechanism for the diphenolase activity (Rodríguez-López, Tudela, Varón, García-Carmona & García-Cánovas, 1992; Ros, Rodríguez-López & García-Cánovas, 1994b; Espín, Morales, Varón, Tudela & García-Cánovas, 1995; Espín, Morales, Varón, Tudela & García-Cánovas, 1997) predicts a linear accumulation of *o*-quinone in the assay medium with a constant rate from the beginning of the kinetic assay (Fig. 1, curve a). If the accumulation of *o*-quinone is not linear, this can be due, for instance, to substrate depletion (either *o*-diphenol or oxygen), instability of *o*-quinone (polymerisation to melanins), or the presence of slow-binding inhibitors. The spectrophotometric recordings obtained in the presence of agaritine could be misunderstood suggesting a time-dependent inhibition by a slow-binding inhibitor with a decrease in the initial velocity to a steady-state inhibited velocity (Fig. 1, curve b). Therefore, these experiments call for caution when nucleophilic compounds are studied in the presence of *o*-quinones (Sugumaran, 1995). Possible artefacts could arise from these experiments due to the nucleophilic power of some compounds, which attack the *o*-quinones generated in the medium. By changing the ratio *t*BC: agaritine conditions (Fig. 1, curves c, d) a decrease in the absorbance can be observed. *t*BQ was very stable in the pH interval from 5 to 8. Therefore, the shape of the spectrophotometric recordings could be explained by the presence of two overlapping reactions (formation and decomposition of *o*-quinone). Depending on the assay conditions the formation or decomposition reaction will prevail.

The *o*-quinones from several *o*-diphenols (*t*BC, catechol, 4-methylcatechol, L-DOPA (3,4-dihydroxyphenylalanine), γ-glutammyl-3,4-dihydroxybenzene (GDHB) and chlorogenic acid) were assayed to study the effect of agaritine on their corresponding *o*-quinones. The study of the effect of agaritine on *o*-quinones from typical endogenous phenolic compounds in mushroom (L-DOPA (3,4-dihydroxyphenylalanine), γ-glutammyl-3,4-dihydroxybenzene (GDHB)) was checked but it could not be systematically studied due to the high instability of these *o*-quinones. In these cases, the *o*-quinones evolved to render melanins very quickly. Therefore, *t*BQ, which was very stable, was used for the characterisation of the effect of agaritine without any artefact due to the polymerisation of the *o*-quinone.

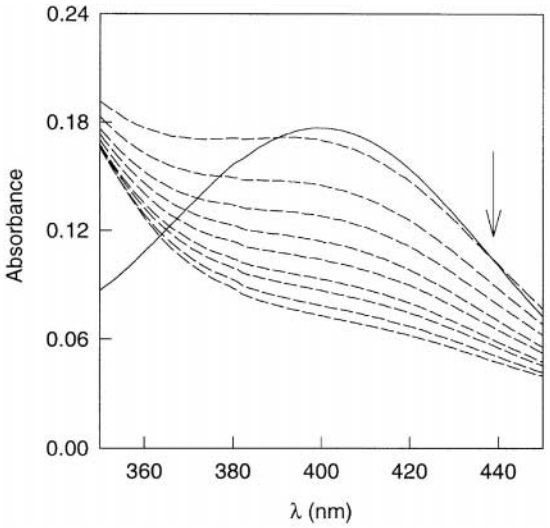


Fig. 2. Scan spectra for the disappearance of *t*BQ in the presence of agaritine. The reaction medium contained 50 mM PB pH 6.8, *t*BC 3 mM, and 0.15 mM $NaIO_4$: (solid trace) without AG; (dashed traces) with 1.5 mM AG. Time between recordings (in direction of arrows) 60 s.

The prevention of melanin formation by agaritine could be corroborated by following the disappearance of the *t*BQ spectrum (Fig. 2). This effect has been already reported for kojic acid (Chen et al., 1991a; Kahn et al., 1995) and azide (Sugumaran, 1995). In the case of agaritine, the disappearance of *o*-quinone followed first order kinetics through a decreasing uniexponential (Fig. 3). Experimental recordings were fitted by non-linear regression to the equation:

$$[X] = [Q]_0 e^{-k_q t} + [QH^+],$$

where X is the sum of the species $QH^+ + Q$ in a slow equilibrium, and $[QH^+]$ is the remaining protonated *o*-quinone in the medium which does not react with agaritine. From the non-linear regression fits to the experimental data, k_q values (the apparent rate constant for the disappearance of *o*-quinone from the medium), $[Q]_0$ (the *o*-quinone concentration depleted from the medium, $[Q]_0 = Q$) and $[QH^+]$ could be estimated.

2.1. Assay conditions

The dependencies of k_q values and $[Q]_0$ as a function of proton, *o*-quinone and agaritine concentrations were studied. For this purpose, *t*BQ was chemically generated in the medium by adding sodium-*meta*-periodate ($NaIO_4$), but keeping the initial concentration of *o*-diphenol (*t*BC) in excess. Under these assay conditions, *t*BQ was very stable at every pH studied. The initial *o*-quinone concentration was produced by adding different $NaIO_4$ concentrations. The *o*-quinone was instantaneously generated in the medium, much faster than if it is generated by adding tyrosinase. This approach was followed to study the net effect of the capability of agaritine for removing the *o*-quinone from the assay medium. Experiments were carried out at pH 6.8 (which is very close to the pH of a mushroom extract in water and also close to the pK_{an} calculated for agaritine), therefore the effective (reactive, deprotonated) agaritine was approximately the half of the initially added ($AG + AGH^+$), but always in excess with regard to the *o*-quinone generated in the medium to make sure that agaritine was not limiting in the reaction.

2.2. Kinetic analysis

A possible reaction mechanism which summarises the effect of agaritine in the prevention of melanin formation is proposed (Scheme 1).

The removal of O_2 from the assay medium by bubbling N_2 did not modify the rate of disappearance of *o*-quinone which meant that oxygen was not involved in the formation of the final colourless product (2-amino-4-[*N*′-(2-*tert*-butyl-4,5-dihydroxyphenyl)-*N*′-(4-hydroxymethyl-phenyl)-hydrazono-carbonyl]-butyric acid). The spectrum of the reaction did not involve the regeneration of *o*-diphenol in the medium. Two isosbestic points were observed at 270 nm and 291 nm which meant that the reaction followed a defined stoichiometry (results not shown). These findings suggest that the reaction is an addition of agaritine to the *o*-quinone and the final product is not oxidised by the *o*-quinone. This is supported by the low reactivity of *t*BQ in experiments to further oxidise the adduct formed when this *o*-quinone was assayed with another nucleophile (3-methyl-2-benzothiazolinone hydrazone)

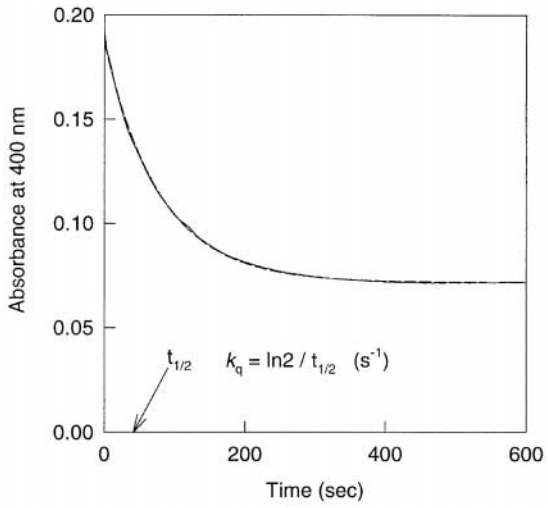
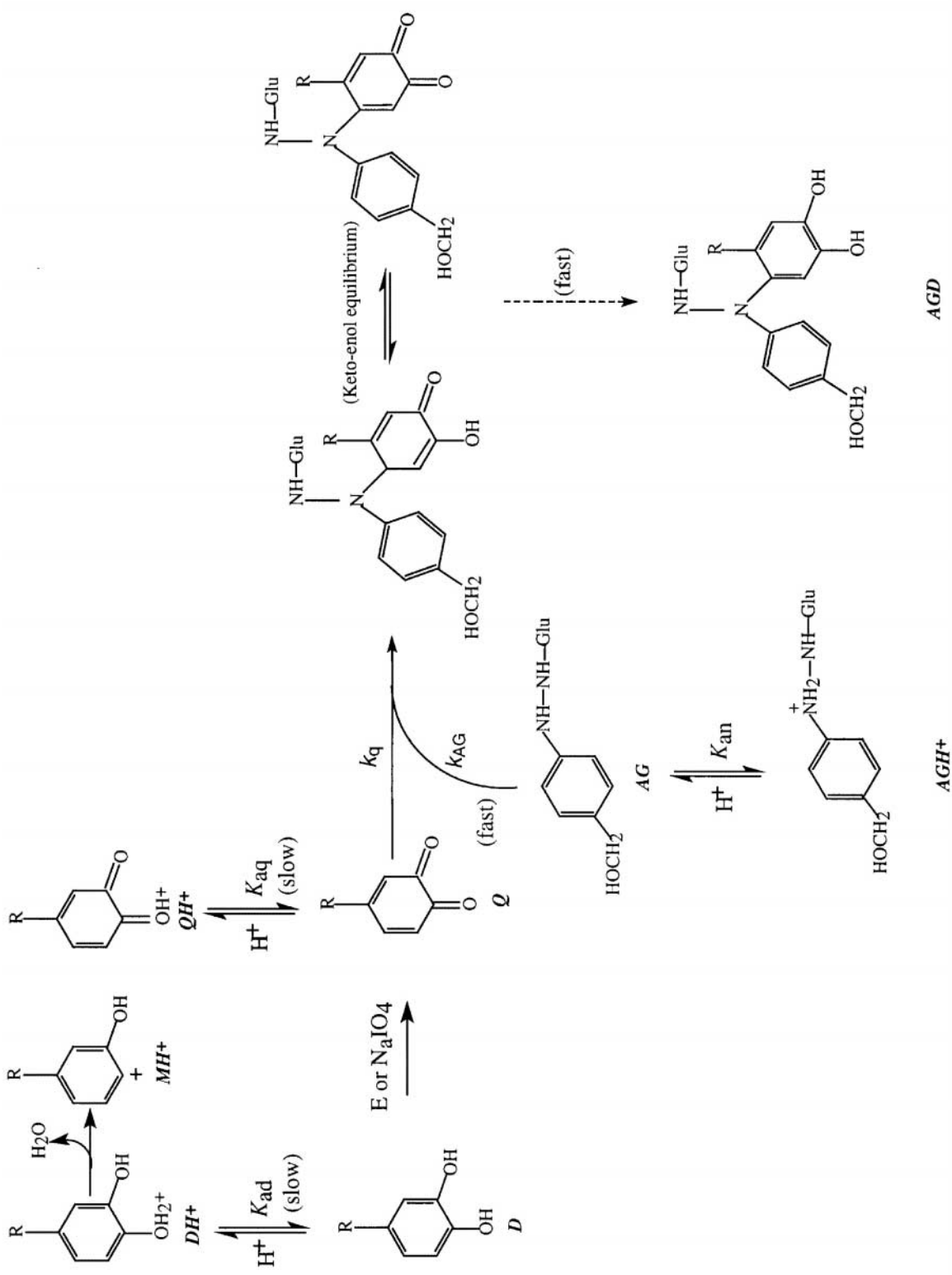


Fig. 3. Experimental recording of the disappearance of *o*-quinone in the presence of agaritine. The reaction medium contained PB 50 mM pH 6.8, *t*BC 3 mM, 0.15 mM $NaIO_4$ and 1.5 mM AG. (———) Experimental data. (– – – –) Non-linear regression fit of experimental data to a decreasing uniexponential equation.



Scheme 1. Structural reaction mechanism to explain the attack of agaritine to *o*-quinones. *D*, deprotonated *o*-diphenol (*t*BC) in equilibrium with DH^+, protonated *o*-diphenol (tBCH$^+$); E, tyrosinase or laccase or peroxidase; $NaIO_4$, sodium-*meta*-periodate; MH^+, protonated *para* or *meta* monophenol; *Q*, deprotonated *o*-quinone (*t*BQ) in equilibrium with QH^+, protonated *o*-quinone (tBQH$^+$); *AG*, deprotonated agaritine in equilibrium with AGH^+, protonated agaritine; *AGD*, final colourless product; Glu, glutamic acid; R, *tert*-butyl.

(Espín et al., 1995). With other more reactive *o*-quinones it could be possible a further oxidoreduction reaction with oxidation of the product AGD by the *o*-quinone to render a possible final adduct agaritine–quinone but also colourless.

Considering Scheme 1, the sum of the species QH^+ and Q can be considered in equilibrium as the species [X] and the sum of the species AG and AGH^+ as the species [Y]:

$$-\frac{d[X]}{dt} = k_{AG}[Y]f_Q[X]f_{AG}[Y] \quad (1)$$

$$f_Q = \frac{K_{aq}}{K_{aq} + [H^+]_0} \quad (2)$$

$$f_{AG} = \frac{K_{an}}{K_{an} + [H^+]_0} \quad (3)$$

integrating, Eq. (1) becomes:

$$[X] = [Q]_0 e^{-\left(k_{AG}[Y]\frac{K_{aq}}{K_{aq}+[H^+]_0}\frac{K_{an}}{K_{an}+[H^+]_0}\right)t} + [QH^+] \quad (4)$$

The removal of *o*-quinone by agaritine did not follow a cuadratic dependence on proton concentration but first-order kinetics through a single unidirectional exponential (thus it can be represented as $[X] = [Q]_0 e^{-k_q t} + [QH^+]$), (Fig. 3). Therefore, in the disappearance of *o*-quinone and according to the dependencies observed, there was a significant pK_a in the process corresponding to the protonation-deprotonation reaction: $AGH^+ \leftrightarrow AG + H^+$. This was further corroborated by titrating agaritine with an automatic titrater. Thus the apparent constant of this transformation (k_q) becomes:

$$k_q = k_{AG}[Y]\frac{K_{an}}{K_{an} + [H^+]_0} \quad (5)$$

2.3. Effect of proton concentration

Inverting Eq. (5) gives:

$$\frac{1}{k_q} = \frac{1}{k_{AG}[Y]} + \frac{1}{k_{AG}[Y]K_{an}}[H^+]_0 \quad (6)$$

Linear regression fitting of $1/k_q$ vs. $[H^+]_0$ (Fig. 4A) gives $K_{an} = (2.1 \pm 0.1)\times 10^{-7}$ M. From this datum, the pK_{aq} value for the equilibrium $AGH^+ \leftrightarrow AG + H^+$, can be obtained ($6.6 \pm 0.02$). This value was corroborated by titration with an automatic titrater (6.7 ± 0.1). Therefore considering the pH, this pK_{an} and the Henderson–Hasselbach equation

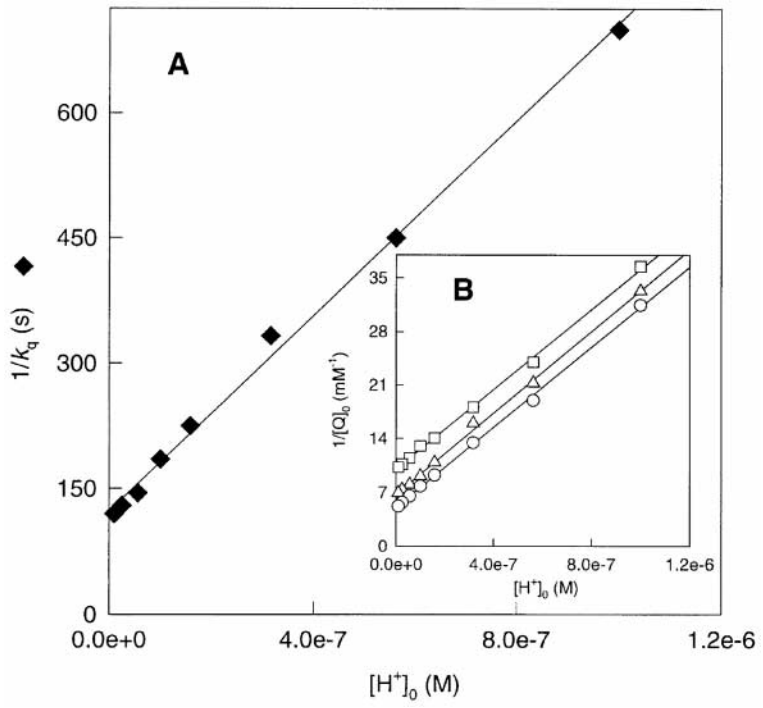


Fig. 4. (A) Dependence of $1/k_q$ vs. initial proton concentration ($[H^+]$). The reaction medium contained PB 50 mM (pH 6 to 8), *t*BC 3 mM, (0.015–0.94) mM AG (depending on the pH) and 0.15 mM $NaIO_4$. (B) $1/[Q]_0$ as function of initial proton concentration ($[H^+]$). The reaction medium was the same as in (A) but $NaIO_4$ (○) 0.2 mM: (△) 0.15 mM and (□) 0.1 mM.

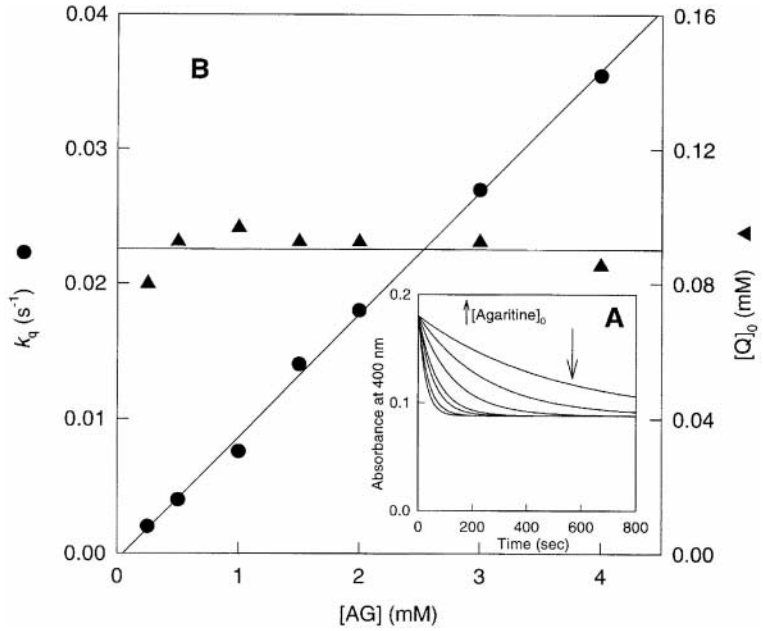


Fig. 5. (A) Spectrophotometric recordings of the disappearance of *o*-quinone with different agaritine concentrations. The reaction medium contained PB 50 mM pH 6.8, *t*BC 3 mM, 0.15 mM $NaIO_4$ and (0.25–4) mM AG. (B) k_q and $[Q]_0$ as a function of initial AG concentration. The reaction medium was the same as in (A).

$$\left(\mathrm{pH} = \mathrm{p}K_{an} + \log\frac{[AG]}{[AGH^+]}\right),$$

the effective (AG, nucleophilic) agaritine concentration for every pH can be calculated.

The intersection value with ordinate $\left(\frac{1}{k_{AG}[Y]}\right)$, gives the value of $k_{AG} = (8.13 \pm 0.4)\ M^{-1}\,s^{-1}$. At this point ($[H^+] \to 0$) all the species of agaritine are in the deprotonated (reactive) state (AG). The protonation–deprotonation of agaritine could explain the observed dependencies of k_q vs. [AG], [X] and pH.

On the other hand, a pK_{aq} value of (6.5 ± 0.08) was calculated with the automatic titrater. Considering the initial *o*-quinone concentration generated in the medium, the pK_{aq} value obtained for the slow equilibrium $QH^+ \leftrightarrow Q + H^+$, and the Henderson–Hasselbach equation

$$\left(\mathrm{pH} = \mathrm{p}K_{aq} + \log\frac{[Q]}{[QH^+]}\right),$$

the concentration of the species Q and QH^+ for every pH can be calculated. Taking into account,

$$K_{aq} = \frac{[Q][H^+]}{[QH^+]} \tag{7}$$

the plot of $1/[Q]_0$ vs. $[H^+]$ (Fig. 4B) gives parallel curves with the slope $1/K_{aq}[QH^+]$ and the intersect in the ordinate axis (when $[H^+] \to 0$, increasing pH), was equal to $1/[Q]_0$ because in this case, all the *o*-quinone species was as the species Q and all the agaritine was as the species AG, and thus all the *o*-quinone was removed from the assay medium. When $[H^+] \to \infty$ (decreasing pH) there are no species Q in the medium (only species QH^+) and also agaritine is protonated (AGH^+) (no reactive).

2.4. Effect of agaritine concentration

At constant pH, *t*BQ was generated in the medium and the effect of various agaritine concentrations was studied. Under these assay conditions, a series of curves was obtained (Fig. 5A) where k_q increased linearly and $[Q]_0$ remained constant when agaritine concentration increased (Fig. 5B). This is consistent with Scheme 1. Considering Eq. (5), at constant pH, the dependence of k_q vs. agaritine concentration was linear, with slope $= k_{AG}C$, where $C = \frac{K_{an}}{K_{an}+[H^+]_0}$ at each pH. The consumed amount of *o*-quinone was independent of the agaritine concentration because its term does not appear in Eq. (5). At constant pH (Eq. (7)), $[Q]_0$ is constant (Fig. 5B). The effective agaritine concentration (AG) at constant pH could be calculated by the Henderson–Hasselbach equation. These experiments where carried out at pH 6.8 and therefore the effective agaritine concentration (AG) was approximately the half of the initial agaritine concentration added.

2.5. Effect of o-quinone concentration

When the initial *o*-quinone concentration was increased ([X]), a family of curves similar to those obtained when the effect of agaritine concentration was studied, was observed (results not shown). In this

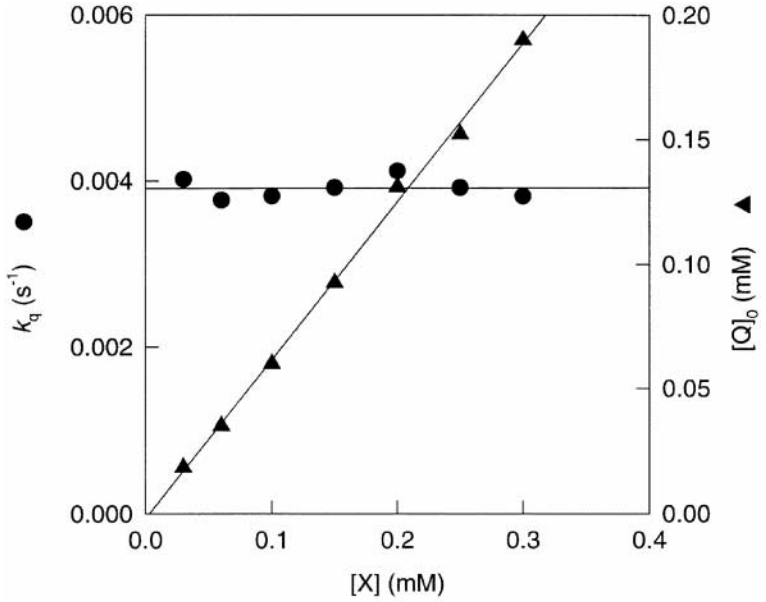


Fig. 6. Dependence of k_q and $[Q]_0$ on initial *o*-quinone concentration ([X]). The reaction medium contained PB 50 mM pH 6.8, *t*BC 3 mM, 0.5 mM AG and (0.03–0.3) mM $NaIO_4$.

case, the rate of disappearance of the *o*-quinone was the same (k_q constant), but $[Q]_0$ increased linearly (Fig. 6). At constant pH, when [X] was raised, taking into account Eq. (7), $[Q]_0$ increased linearly through the origin and with slope of $\frac{K_{aq}}{[H^+]_0}$ at each pH.

3. Conclusions

Agaritine is an abundant and characteristic compound of *Agaricus bisporus*. This phenylhydrazyne is a nucleophile, which is able to attack *o*-quinones depending on its protonation state. It is known that *o*-quinones are very reactive and have bacteriostatic activity (Pierpoint, Ireland & Carpenter, 1997). The browning reaction catalysed by tyrosinase may be involved in a defensive mechanism against bacterial infections (Soler-Rivas, Arpin, Olivier & Wichers, 1997). Agaritine could play *in vivo* a role as endogenous regulator of the *o*-quinone concentration formed in *A. bisporus* with different degrees of efficiency depending on the microenvironmental conditions of the fungus (Figs. 4–6).

On the other hand, this study calls for caution in the use of agaritine in experiments with *o*-quinone accumulation because artefacts can arise from these experiments due to the effect of agaritine on *o*-quinones.

Taking into account the effect of agaritine to react with *o*-quinones to render colourless products, the synthesis of agaritine-related compounds could be helpful in the prevention of melanin formation in fruits and vegetables.

4. Experimental

4.1. Chemicals

Catechol, 4-methylcatechol, L-DOPA, 4-*t*-butylcatechol and chlorogenic acid were purchased from Fluka. All other reagents were of analytical grade and supplied by Merck. Stock solutions of phenolic compounds were prepared in 0.15 mM *o*-phosphoric acid as solvent to prevent autoxidation. Milli-Q system (Millipore Corp., U.S.A.) ultrapure water was used throughout this research. 50 mM sodium phosphate buffer (PB) (pH 6–8) was used.

4.2. Enzyme source

Mushroom (*Agaricus bisporus*) tyrosinase (3900 units/mg) was purchased from Sigma. The commercial preparation of tyrosinase contained a single isoenzyme with an isoelectric point around 4.1 determined by isoelectric focusing and a major band of M_r 43,000 in SDS–PAGE electrophoresis (results not shown).

4.3. Isolation and purification of agaritine

Agaritine was extracted from the gills of fresh *A. bisporus* fruitbodies U1 stage 3 according to Hammond and Nichols (1976). 100 mg of gills were ground three times in a blender with 200 ml of 0.5% (w/v) sodium bisulfite in 1% acetic acid. Homogenates were filtered and centrifuged for 20 min at 10,000*g*. Supernatants were pooled and the crude extract was taken to dryness; dry material was then dissolved into

5 ml distilled water and loaded on the chromatographic column. The exchange ion resins were prepared as described by Redgwell (1980). The anion exchanger QAE Sephadex A-25 was activated two days in 0.5 M sodium formate and after filtration, stored in 0.05 M sodium formate. The anion exchange column (80×2.5 cm) was eluted with water (1 ml×min^{-1}) allowing a coarse separation of agaritine. Spectrum of the eluted fractions was scanned in a range 200–400 nm. Fractions containing the phenylhydrazine (λ_{max} = 237 nm) were pooled and taken to dryness. The dry residue was suspended in distilled water and injected on an HPLC column Lichrosorb (25×2.5 cm, particle size 100 Å), RP C18 eluted with methanol (MeOH) 10% at a flow rate of 2 ml×min^{-1}. Fractions containing agaritine were pooled and taken to dryness. Agaritine was crystallised from hot ethanol. Purity was monitored by HPLC using a Nucleosil (25×0.5 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^{-1}.

4.4. Titration assays

The compounds *t*BC, *t*BQ and agaritine were in different degrees of protonation depending on the pH. The ionization constant for the groups responsible of the existence of these protonation-deprotonation equilibrium were determined in the pH range 3 to 8 with a 718 Stat Titrino™ titrater. The different pK_a values were obtained: (5.5 ± 0.07) for the equilibrium $t\mathrm{BCH}^+ \leftrightarrow t\mathrm{BC} + \mathrm{H}^+$; ($6.5 \pm 0.08$) for $t\mathrm{BQH}^+ \leftrightarrow t\mathrm{BQ} + \mathrm{H}^+$ and (6.7 ± 0.1) for $\mathrm{AGH}^+ \leftrightarrow \mathrm{AG} + \mathrm{H}^+$.

4.5. Spectrophotometric assays

Absorption spectra were recorded in an Perkin Elmer Lambda-2 spectrophotometer, on-line interfaced to a 486-DX33 microcomputer. Temperature was controlled at 25° with a circulating bath with heater/cooler and checked using a precision of ±0.1°. Kinetic assays were also carried out with the above equipment by measuring the appearance or disappearance of *o*-quinones (*t*BQ) at 400 nm (Waite, 1976) in the assay medium. Reference cuvettes contained all the components except the substrate in a final volume of 1 ml.

4.6. Kinetic data analysis

All the experiments were carried out in duplicate with 500 data points per instrumental recording. Mean values of the appropriate kinetic parameters are shown in the Figures, whereas the reciprocals of their variances were used as weighting factors in further statistical analysis. Data fittings to linear and non-linear regression (Leatherbarrow, 1990; Brand, & Johnson, 1992) using an improved Gauss–Newton algorithm implemented in the Sigma Plot™ program for Windows™ (Jandel Scientific, 1994).

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