Kinetics of Activation of Latent Mushroom (*Agaricus bisporus*) Tyrosinase by Benzyl Alcohol

Juan Carlos Espín* and Harry J. Wichers

Agrotechnological Research Institute (ATO-DLO), Bornsesteeg 59, P.O. Box 17, 6700 AA Wageningen, The Netherlands

A latent isoform of *Agaricus bisporus* tyrosinase has been isolated and activated by benzyl alcohol, one of the major volatile compounds in mushrooms of this genus. The progress curve that describes the activation process reached the steady-state rate (V_{ss}) after a lag period (τ). The rate of active tyrosinase formation was calculated by coupling the oxidation of *o*-diphenols to the activation process. V_{ss} depended on benzyl alcohol, *o*-diphenol, and latent tyrosinase concentrations. The lag period depended on benzyl alcohol concentrations but not on *o*-diphenol and enzyme concentrations. The size of the latent mushroom tyrosinase was 67 kDa, determined by SDS−PAGE and Western blotting assays. This size was not modified after activation by benzyl alcohol. The presence of a lag period and the lack of change of the molecular mass of the protein after activation could indicate a slow conformational change of the protein to render the final active form. The values of the kinetic constants V_{max} and K_m on the *o*-diphenols 4-*tert*-butylcatechol, L-DOPA, and dopamine were different between the latent tyrosinase activated by benzyl alcohol and the commercial tyrosinase. They might indicate that a different final active tyrosinase, depending on the activator used, could arise.

Keywords: *Agaricus; activation; benzyl alcohol; latent; mushroom; tyrosinase*

INTRODUCTION

Tyrosinase or polyphenol oxidase (EC 1.14.18.1, PPO) as present in plant tissues plays an important role in the quality of fruit and vegetable processing and during storage of the processed foods. Prevention of browning in foods, enzymatic or nonenzymatic, has long been the concern of food scientists (Dawley and Flurkey, 1993; Kahn and Zakin, 1995; Espín et al., 1998a). Tyrosinase is a copper-containing enzyme that, in the presence of molecular oxygen, 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, nonenzymatically polymerize to render brown, black, or red pigments (melanins) (Prota, 1988; Martínez and Whitaker, 1995).

The enzyme tyrosinase can be found as either latent or active form (Whitaker, 1995). Latent tyrosinase represents >95% of total tyrosinase activity in mushrooms (Yamaguchi et al., 1970; van Leeuwen and Wichers, 1999). Active tyrosinase is the major factor responsible in the enzymatic browning of mushrooms, causing severe economical losses to mushroom growers.

Latent tyrosinase from many sources can be activated by different treatments or agents, which include detergents (Moore and Flurkey, 1990; Nellaiappan and Sugumaran, 1996), acid shock (Kenten, 1957), fatty acids (Sugumaran and Nellaiappan, 1991), alcohols (Asada et al., 1993), proteases (King and Flurkey, 1987; Robinson and Dry, 1992; Chosa et al., 1997), and pathogen attack (Soler-Rivas et al., 1997).

The aim of the work presented here is to study the kinetics for the activation of a latent mushroom tyrosinase by benzyl alcohol, one of the most abundant endogenous volatile compounds in *Agaricus bisporus* mushroom.

* Author to whom correspondence should be addressed (fax 31-317-475347; e-mail J.C.ESPIN@ato.dlo.nl).

MATERIALS AND METHODS

Reagents. 4-*tert*-Butylcatechol (TBC), L-DOPA, dopamine, and tyramine were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade and also supplied by Sigma. Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this research.

Preparation of Commercial Tyrosinase. Fluka (1200 units/mg) tyrosinase was purified by using an anion exchange column (DEAE-Sepharose Fast Flow, length = 75 cm, diameter = 5 cm; Pharmacia, Uppsala, Sweden). The column was equilibrated with 20 mM Bis-Tris buffer (pH 6). A stepwise gradient of increasing sodium chloride (NaCl) concentrations was applied (3 mL/min). One major isoform with an isoelectric point of 4.3 was isolated. Fractions with this isoform, dialyzed and concentrated, were used as the source of commercial enzyme.

Preparation of a Latent Mushroom Tyrosinase. Boxes with U1-spawned compost and casing soil from the Mushroom Experimental Station (Horst, The Netherlands) were transported after bud initiation to a climate room at ATO-DLO (18 °C, 80% relative humidity). Mushrooms were harvested at stage 5 and frozen in liquid nitrogen immediately after picking. The developmental stage was determined according to the procedure of Hammond and Nichols (1976). The freeze-dried mushrooms were ground under liquid nitrogen to a fine powder with a mortar and pestle. The powder was rehydrated with 10 mM sodium phosphate buffer (PB) containing 10 mM ascorbic acid and mixed thoroughly on a vortex shaker. This homogenate was then extracted for 25 min on ice and centrifuged at 12000*g* for 10 min.

The supernatant was immediately applied to the same column above-described. The column was also equilibrated with 20 mM Bis-Tris buffer (pH 6). A stepwise gradient of increasing sodium chloride (NaCl) concentrations was applied (3 mL/min). The different fractions were assayed with TBC in

10.1021/jf981334z CCC: $18.00
Published on Web 08/17/1999

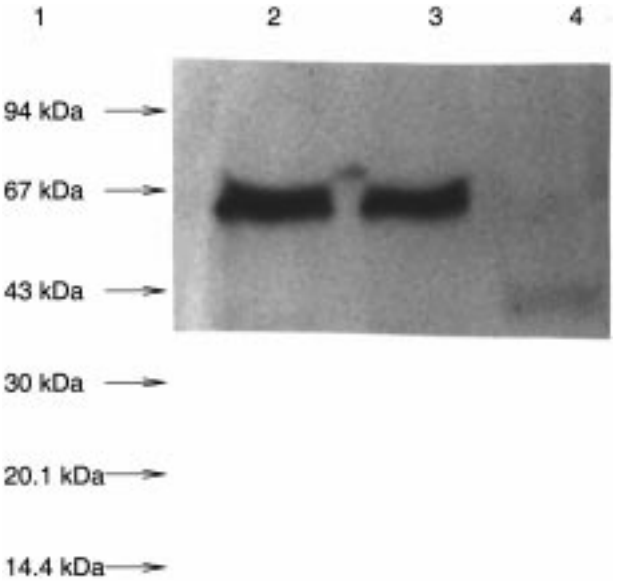

Figure 1. Identification of mushroom tyrosinase by Western blotting on SDS−PAGE: lane 1, markers; lane 2, latent mushroom tyrosinase (10 μg/mL); lane 3, mushroom tyrosinase (10 μg/mL) activated by BA (0.3 M); lane 4, commercial (Fluka) mushroom tyrosinase (1 μg/mL). See Materials and Methods for details.

the absence and presence of SDS to discriminate between active and latent tyrosinase isoforms. A major latent isoform with an isoelectric point of 5.6 (as determined by analytical isoelectric focusing, results not shown) was eluted at 50 mM NaCl concentration. The proportion of latent tyrosinase (100%) was determined by comparing the activities in the presence and absence of SDS. This latent tyrosinase isoform showed a band of 67 kDa determined by SDS−PAGE and Western blotting analysis (Figure 1). The existence of this 67 kDa latent mushroom tyrosinase has not been previously reported. This finding agrees with the previously reported putative tyrosinase cDNA clone, which encoded a protein of ~64 kDa (Wichers et al., 1995; van Gelder et al., 1997).

Electrophoresis. SDS−PAGE experiments were performed under denaturing conditions in 10% polyacrylamide gels with a minigel Bio-Rad system. Samples were diluted with 50 mM TCB (pH 7), containing 0.5 mM β-mercaptoethanol, 2% SDS, 1% bromophenol blue, and 10% glycerol. Electrophoresis was conducted at a constant voltage of 200 V in a buffer (pH 6.8) containing 3 g/L Tris base, 14.4 g/L glycine, and 1 g/L SDS. For molecular mass determination, the calibration kit of SDS−PAGE standards (low range of M_r) from Bio-Rad was used. After electrophoresis, the gels were equilibrated for 30 min at 4 °C in transfer buffer (TB) containing 3 g/L Tris base, 14.4 g/L glycine, and 20% methanol (v/v). The gels were then blotted onto a PVDF membrane for 1 h at a constant voltage of 100 V in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The ECL protocol (Amersham Int., England) was followed to develop tyrosinase bands on the gel. This method is based on light emission for detection of immobilized specific antigens. After electroblotting, the membrane was rinsed in TBS and incubated in the block solution (low-fat dried milk) for 1 h. After incubation, the membrane was washed in TTBS for 25 min and incubated with the first antibody (anti-AbPPO) diluted in TTBS solution for 2 h. After incubation with anti-AbPPO, the membrane was washed in TTBS for 25 min and then incubated with the secondary antibody (HRP-Ab) diluted in TTBS solution for 1 h. After incubation with HRP-Ab, the membrane was washed in TTBS for 35 min. To detect the bands, the reagents of the kit were mixed (1:1) according to the manufacturer's instructions. After 1 min of incubation, the membrane was immediately exposed to a photographic film for 1 min.

Enzymatic Assays. Tyrosinase activity on TBC was determined at 400 nm by measuring the accumulation of 4-(*tert*-butyl)benzo-1,2-quinone (TBQ). This *o*-quinone was highly stable at every pH assayed and for a longer period than those used in the activity measurements (Waite, 1976; Ros et al., 1994a). The final volume of assay was 1 mL. One unit of active form of tyrosinase was defined as the amount of the enzyme that produces 1 μmol of TBQ per minute.

Tyrosinase activities on L-DOPA and dopamine were determined by measuring dopachrome and dopaminechrome accumulations at 475 and 480 nm, respectively (Ros et al., 1994b).

N,*N*′-Dimethylformamide (DMF) (1%, v/v) was added to the assay medium to enhance the solubility of benzyl alcohol (BA). This proportion of DMF did not alter kinetic properties of tyrosinase (Espín et al., 1995a, 1997a, 1998b).

The spectrophotometric assays were recorded in an ultraviolet−visible Perkin-Elmer Lambda-2 spectrophotometer (Überlingen, Germany), on-line interfaced to a Pentium-100 microcomputer (Ede, The Netherlands). Temperature was controlled at 25 °C with a circulating bath with a heater/cooler and checked using a precision of ±0.1 °C.

Kinetic Data Analysis. The values of K_m and V_{max} were calculated from triplicate measurements of the steady-state rate, V_{ss}, for each initial substrate concentration ($[S]_0$). The reciprocals of the variances of V_{ss} were used as weighting factors to the nonlinear regression fitting of V_{ss} versus $[S]_0$ to the Michaelis equation (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).

Other Methods. Protein content was determined by using the method of Bradford (1976) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

The activation of latent tyrosinase by some alcohols has been previously reported (Asada et al., 1993). However, the activation of latent mushroom tyrosinase by volatile compounds such as BA has not been previously published.

The activation of latent mushroom tyrosinase by BA was characterized by the presence of a lag period (τ) prior to the attainment of the steady-state rate (V_{ss}) (Figure 2A). The molecular weight of the latent tyrosinase (67 kDa) did not change after activation by BA (Figure 1). The presence of the transient phase in the activation process suggested that the activation could take place through a slow conformational change of the enzyme to render the active tyrosinase. Moreover, V_{ss} depended on BA concentrations with a sigmoid pattern (Figure 2B), which also happens in the activation of other latent tyrosinases by SDS (Moore and Flurkey, 1990; Nellaiappan and Sugumaran, 1996). This could indicate a possible detergent-like character of BA, which could explain the activation of the latent enzyme by means of a conformational change of the protein, similar to that caused by SDS. The lag period of the activation process diminished in a sigmoid way with increasing BA concentrations. τ decreased to zero at certain BA concentrations, but a concomitant decrease of V_{ss} was obtained (Figure 2A,B).

The optimum BA concentration ($[BA]_{opt}$) to activate the latent enzyme varied in a sigmoid manner with pH. The $[BA]_{opt}$ was maximum, 0.3 M (3% of BA v/v), at pH >6.5 (Figure 3) because protonation of the latent enzyme by acid shock could facilitate the activation process at low pH. V_{ss} changed in a sigmoid way and τ increased with pH (Figure 4). The profile of the optimum pH curve was equal to that previously observed for commercial tyrosinase (Espín et al., 1997b).

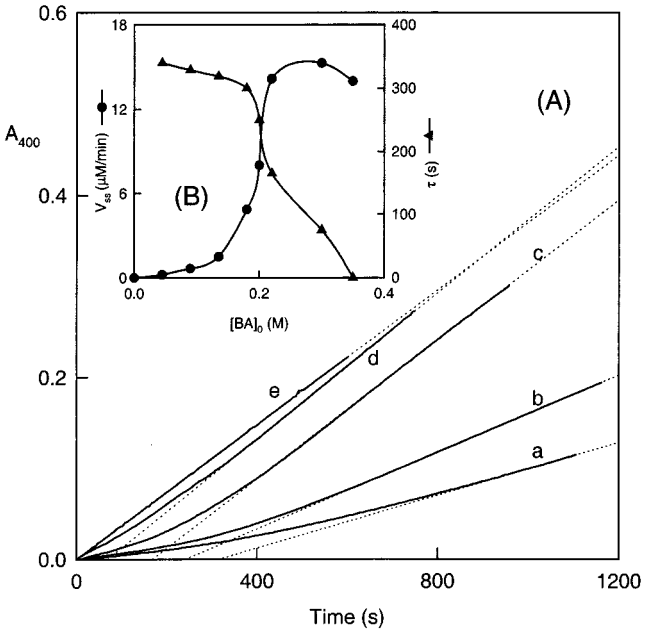

Figure 2. (A) Spectrophotometric recordings for the activation of a latent mushroom tyrosinase isoform by BA. Conditions were as follows: 50 mM PB (pH 6.8), 2.5 mM TBC, 0.07 μg/mL latent tyrosinase, and BA (a) 0.1 M, (b) 0.2 M, (c) 0.25 M, (d) 0.3 M, and (e) 0.35 M. (B) Dependence of V_{ss} (●) and τ (▲) on $[BA]_0$. Conditions were as in Figure 2A.

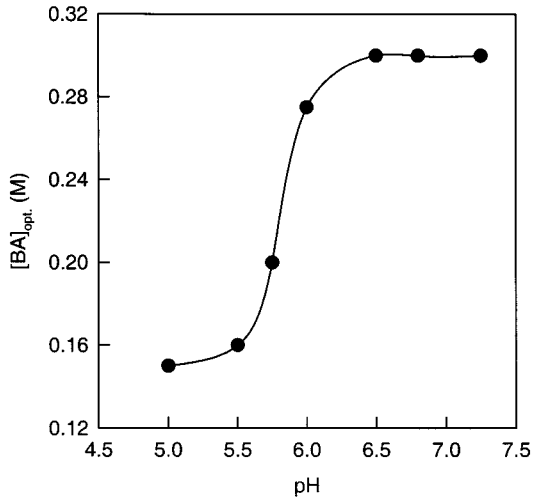

Figure 3. Dependence of optimum BA concentration ($[BA]_{opt}$) on pH. Conditions were as follows: 50 mM AB (pH 5 and 5.5), 50 mM PB (pH 5.75−7.25), 2.5 mM TBC, and 0.035 μg/mL latent mushroom tyrosinase.

V_{ss} was linearly dependent and τ remained constant with varying latent enzyme concentrations (Figure 5). Furthermore, V_{ss} was hyperbolically dependent and τ remained constant on *o*-diphenol concentration (Figure 6).

K_m values changed in a hyperbolic way with pH, whereas V_{max} values were constant (Figure 7; Table 1). This is also the characteristic behavior of commercial tyrosinase, which meant that the protonation of BA-activated tyrosinase at low pH resulted in higher K_m values toward its substrates. This fits to a previously proposed reaction mechanism for tyrosinases from many sources (mushroom, frog epidermis, grape, apple, pear, avocado, artichoke, and strawberry) (Rodríguez-López et al., 1992; Ros et al., 1994b; Espín et al., 1995a,b, 1997a−f, 1998b−e).

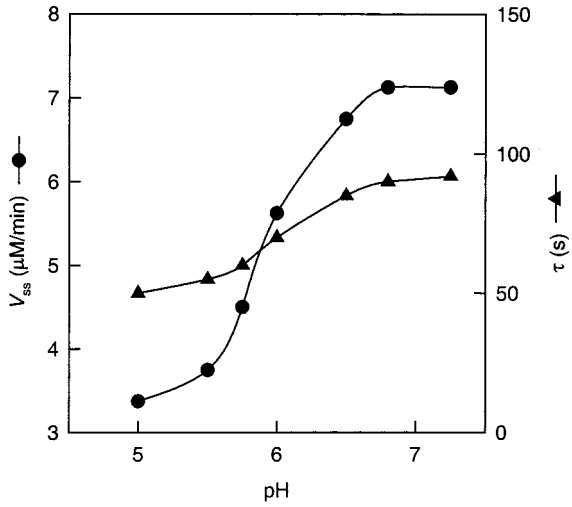

Figure 4. Dependence of V_{ss} (●) and τ (▲) on pH in the activation of a latent mushroom tyrosinase by BA. Conditions were as follows: 50 mM AB (pH 4−5.5), 50 mM PB (pH 5.75−7.25), 2.5 mM TBC, $[BA]_{opt}$ at every pH, and 0.035 μg/mL latent tyrosinase.

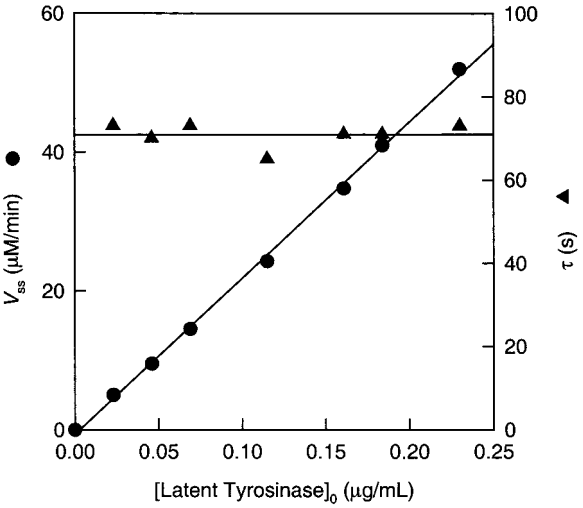

Figure 5. Dependence of V_{ss} (●) and τ (▲) on latent tyrosinase concentration in the activation of latent tyrosinase by BA. Conditions were as follows: 50 mM PB (pH 6.8), 2.5 mM TBC, 0.3 M BA, and 0.035−0.23 μg/mL latent tyrosinase.

The values of the kinetic constants V_{max} and K_m were different between the BA-activated and commercial tyrosinases (Table 1). The observations suggest that the final active tyrosinase has different kinetic properties depending on the activator. For instance, the fully active commercial tyrosinase has a molecular mass of 43 kDa, lower than that for the BA-activated tyrosinase (67 kDa). BA could induce a conformational change in the latent tyrosinase to render the active tyrosinase. Therefore, the mechanism of activation is obviously different for both mushroom tyrosinases. This suggests that different activators render active tyrosinases with different catalytic and affinity properties toward their substrates (Table 1). For instance, K_m values for TBC were higher for commercial tyrosinase (3 mM at pH 6.8) than for BA-activated tyrosinase (2 mM at pH 6.8). However, K_m values for both L-DOPA and dopamine were lower for commercial tyrosinase (0.7 and 1.8 mM, respectively, at pH 6.8) than for BA-activated tyrosinase (4.7 and 5.5, respectively, at pH 6.8) (Table 1). This could be due to the presence of different charges in the active site of the enzymes. Moreover, the sequence of V_{max}

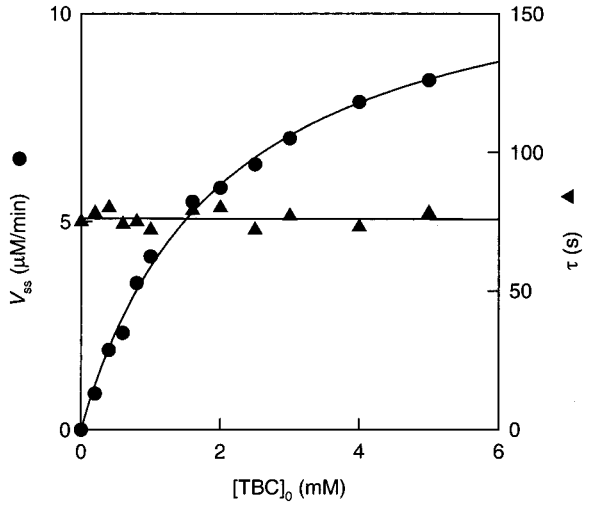

Figure 6. Dependence of V_{ss} (●) and τ (▲) on TBC concentration in the activation of latent tyrosinase by BA. Conditions were as follows: 50 mM PB (pH 6.8), 0.3 M BA, 0.07 μg/mL latent tyrosinase, and 0.2−5 mM TBC.

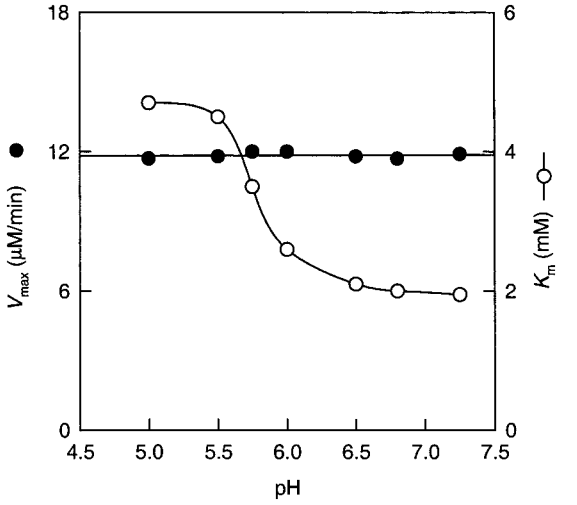

Figure 7. Dependence of V_{max} (●) and K_m (▲) on pH for the BA-activated tyrosinase isoform. Conditions were as follows: 50 mM AB (pH 5, 5.5), 50 mM PB (pH 5.75−7.25), 2.5 mM TBC, $[BA]_{opt}$ at every pH, and 0.035 μg/mL latent tyrosinase.

values (TBC > dopamine > L-DOPA) was always the same at every pH and for both tyrosinase isoforms. This sequence could be explained on the basis of a quantitative effect of the ring substituent on the rate of phenolic compound oxidation catalyzed by several tyrosinases previously reported (Espín et al., 1998b−e). This effect is related to the capacity of the side chain of the different phenolic compounds to donate electrons toward the aromatic ring as well as to the molecular size of this side chain (Espín et al., 1998b−e).

When latent tyrosinase was activated with higher or lower BA concentration than the optimum, the kinetic constants V_{max} and K_m on TBC were different. V_{max} = 9.5 μM/min and K_m = 3.2 mM when the latent enzyme was activated with 0.35 M BA, V_{max} = 1.5 μM/min and K_m = 5.2 mM when activated with 0.1 M BA, and V_{max} = 11.8 μM/min and K_m = 2 mM at the optimum BA concentration (0.3 M). This indicates that if higher benzyl concentration is used for the activation, the unfolding of the enzyme is very fast (no lag period) but the catalytic efficiency of the final active form is worse (lower V_{max} and higher K_m values) than when the optimum BA concentration is used. At lower BA concentration (long lag period), the active site of the enzyme is also somehow different and less available to the substrate, and then the active enzyme also shows lower V_{max} and higher K_m values.

Table 1. Values of the Kinetic Constants V_{max} and K_m for BA-Activated and Commercial Mushroom Tyrosinase Isoforms[a]

		BA-activated isoform		commercial tyrosinase	
substrate	pH	V_{max} (μM/min)	K_m (mM)	V_{max} (μM/min)	K_m (mM)
TBC[b]	5	11.8 ± 0.81	4.7 ± 0.31	7.9 ± 0.45	5.6 ± 0.43
L-DOPA[c]		1.4 ± 0.05	8.3 ± 0.48	1.8 ± 0.09	2.7 ± 0.20
dopamine[c]		5.5 ± 0.21	8.4 ± 0.45	7.2 ± 0.41	3.8 ± 0.38
TBC	5.5	12.1 ± 0.75	4.5 ± 0.26	8.0 ± 0.40	5.4 ± 0.41
L-DOPA		1.6 ± 0.05	8.1 ± 0.40	1.9 ± 0.10	2.5 ± 0.28
dopamine		5.8 ± 0.30	7.9 ± 0.40	7.4 ± 0.51	3.6 ± 0.31
TBC	5.75	12.0 ± 0.79	3.5 ± 0.25	8.3 ± 0.41	5.1 ± 0.31
L-DOPA		1.5 ± 0.06	6.0 ± 0.30	1.7 ± 0.09	2.2 ± 0.21
dopamine		5.5 ± 0.24	6.5 ± 0.42	7.3 ± 0.41	3.3 ± 0.25
TBC	6	11.8 ± 0.69	2.6 ± 0.21	8.0 ± 0.50	3.8 ± 0.41
L-DOPA		1.6 ± 0.06	5.1 ± 0.35	1.8 ± 0.08	1.2 ± 0.08
dopamine		5.5 ± 0.25	5.8 ± 0.40	7.2 ± 0.51	2.3 ± 0.19
TBC	6.5	11.9 ± 0.89	2.1 ± 0.19	8.2 ± 0.40	3.3 ± 0.21
L-DOPA		1.4 ± 0.05	4.7 ± 0.25	1.8 ± 0.09	0.8 ± 0.41
dopamine		5.5 ± 0.36	5.4 ± 0.35	7.1 ± 0.50	1.8 ± 0.15
TBC	6.8	11.9 ± 0.79	2.0 ± 0.17	8.0 ± 0.41	3.0 ± 0.21
L-DOPA		1.6 ± 0.05	4.7 ± 0.25	1.9 ± 0.12	0.7 ± 0.06
dopamine		5.3 ± 0.31	5.5 ± 0.35	7.2 ± 0.41	1.8 ± 0.14
TBC	7.25	12.1 ± 0.91	1.9 ± 0.13	7.9 ± 0.42	2.9 ± 0.18
L-DOPA		1.5 ± 0.06	4.8 ± 0.31	1.8 ± 0.13	0.7 ± 0.04
dopamine		5.4 ± 0.32	5.3 ± 0.30	7.1 ± 0.39	1.9 ± 0.15

[a] (BA-activated enzyme): 50 mM PB (pH 6.8), $[BA]_{opt}$ at every pH, 0.03 μg/mL latent tyrosinase isoform. (Commercial tyrosinase): 50 mM PB (pH 6.8), 0.05 μg/mL commercial tyrosinase. [b] λ = 400 nm (Waite, 1976). [c] λ_{L-dopa} = 475 nm, $\lambda_{dopamine}$ = 480 nm (Ros et al., 1994b).

To our knowledge there are no previous studies in which this kinetic comparison has been carried out upon activation by alcohols. However, it is known that there are different behaviors of tyrosinase in the absence and presence of SDS regarding inhibitory sensitivity, thermal stability, optimum pH, etc. (Moore and Flurkey, 1990).

It can be suggested that volatile compounds that naturally occur in mushrooms, such as BA, play a role in the activation of latent mushroom tyrosinase. However, the physiological significance of the experiments presented here is not readily apparent. Nevertheless, these experiments have been conducted in the hope that the results will provide some insights into the mechanism of the activation phenomenon. Experiments in modified atmospheres (results not shown) could support the physiological significance of this process. In these experiments, mushrooms in BA gas atmosphere turned brown much more quickly than those with other volatile compounds such as acetone, methanol, or ethanol. This suggests a correlation of the activation of latent tyrosinase by BA (Figures 2−7) and the degree of browning in mushrooms. The high BA concentration used in the in vitro assays (0.3 M, 3% v/v) was necessary to achieve reliable kinetic assay conditions to describe the activation process, because with much lower BA concentrations (experiments in modified atmospheres) it took ~2 days to detect significant activation. Assuming that the formation of BA is correlated to senescence, tyrosinase activation might be an event that is inseparably associated with senescence.

The activation of the latent enzyme by BA might occur through a slow unfolding of the protein to expose the active site. This is consistent with the constant molecular weight after activation and the presence of a lag period prior to the attainment of the steady-state rate.

ABBREVIATIONS USED

AB, sodium acetate buffer; anti-AbPPO, first antibody (polyclonal antibody anti-tyrosinase developed in mouse); BA, benzyl alcohol; $[BA]_0$, initial benzyl alcohol concentration; $[BA]_{opt}$, optimum benzyl alcohol concentration; Bis-Tris, bis[2-hydroxyethyl]iminotris[hydroxymethyl]-methane; DMF, *N*,*N*′-dimethylformamide; L-DOPA, L-3,4-dihydroxyphenylalanine; dopamine, 3,4-dihydroxyphenethylamine; HRP-Ab, secondary antibody (anti-mouse immunoglobulin G conjugated with horseradish peroxidase, developed in goat); K_m, Michaelis constant of active tyrosinase toward TBC; M_r, molecular mass; PB, sodium phosphate buffer; PVDF, Immobilon-P transfer membrane, pore size = 0.45 μm (Millipore); SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; τ, lag period; TB, transfer buffer, pH 8.3; TBC, 4-*tert*-butylcatechol; TBQ, 4-(*tert*-butyl)benzo-1,2-quinone; TCB, tris[hydroxymethyl]aminomethane chlorhidric buffer; Tris, tris-[hydroxymethyl]aminomethane; TTBS, TCB saline (pH 7.5) with 0.05% Tween 20; V_{ss}, steady-state rate; V_{max}, maximum steady-state rate.

ACKNOWLEDGMENT

We are grateful to Dr. Cristina Soler-Rivas from ATO-DLO for her assistance in Western blot assays.

LITERATURE CITED

Asada, N.; Fukumitsu, T.; Fujimoto, K.; Masuda, K.-I. Activation of prophenoloxidase with 2-propanol and other organic compounds in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **1993**, *23*, 515−520.

Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **1983**, *210*, 727−735.

Chosa, N.; Fukumitsu, T.; Fujimoto, K.; Ohnishi, E. Activation of prophenoloxidase A_1 by an activating enzyme in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **1997**, *27*, 61−68.

Dawley, R. M.; Flurkey, W. H. 4-hexylresorcinol, a potent inhibitor of mushroom tyrosinase. *J. Food Sci.* **1993**, *58*, 609−610.

Endrenyi, L. *Kinetic Data Analysis: Design and Analysis of Enzyme and Pharmacokinetics Experiments*; Plenum: New York, 1981.

Espín, J. C.; Morales, M.; Varón, R.; Tudela, J.; García-Cánovas, F. A continuous spectrophotometric method for determining the monophenolase and diphenolase activities of apple polyphenol oxidase. *Anal. Biochem.* **1995a**, *231*, 237−246.

Espín, J. C.; Morales, M.; Varón, R.; Tudela, J.; García-Cánovas, F. Monophenolase activity of polyphenol oxidase from Verdedoncella apple. *J. Agric. Food Chem.* **1995b**, *43*, 2807−2812.

Espín, J. C.; Morales, M.; Varón, R.; Tudela, J.; García-Cánovas, F. Continuous spectrophotometric method for determining the monophenolase and diphenolase activities of pear polyphenol oxidase. *J. Food Sci.* **1996**, *61*, 1177−1181.

Espín, J. C.; Morales, M.; García-Ruiz, P. A.; Tudela, J.; García-Cánovas, F. Improvement of a continuous spectrophotometric method for determining the monophenolase and diphenolase activities of mushroom polyphenol oxidase. *J. Agric. Food Chem.* **1997a**, *45*, 1084−1090.

Espín, J. C.; Varón, R.; Tudela, J.; García-Cánovas, F. Kinetic study of the oxidation of 4-hydroxyanisole catalyzed by tyrosinase. *Biochem. Mol. Biol. Int.* **1997b**, *41*, 1265−1276.

Espín, J. C.; Morales, M.; Varón, R.; Tudela, J.; García-Cánovas, F. Monophenolase activity of polyphenol oxidase from blanquilla pear. *Phytochemistry* **1997c**, *44*, 17−22.

Espín, J. C.; Trujano, M. F.; Tudela, J.; García-Cánovas, F. Monophenolase activity of polyphenol oxidase from Haas avocado. *J. Agric. Food Chem.* **1997d**, *45*, 1091−1096.

Espín, J. C.; Ochoa, M.; Tudela, J.; García-Cánovas, F. Monophenolase activity of strawberry polyphenol oxidase. *Phytochemistry* **1997e**, *45*, 667−670.

Espín, J. C.; Tudela, J.; García-Cánovas, F. Monophenolase activity of polyphenol oxidase from artichoke heads (*Cynara scolymus* L.). *Lebensm.-Wiss. -Technol.* **1997f**, *30*, 819−825.

Espín, J. C.; Jolivet, S.; Wichers, H. J. Inhibition of mushroom polyphenol oxidase by agaritine. *J. Agric. Food Chem.* **1998a**, *46*, 2976−2980.

Espín, J. C.; Tudela, J.; García-Cánovas, F. 4-Hydroxyanisole: the most suitable monophenolic substrate for determining spectrophotometrically the monophenolase activity of polyphenol oxidase from fruits and vegetables. *Anal. Biochem.* **1998b**, *259*, 118−126.

Espín, J. C.; García-Ruiz, P. A.; Tudela, J.; García-Cánovas, F. Study of the stereospecificity in pear and strawberry polyphenol oxidases. *J. Agric. Food. Chem.* **1998c**, *46*, 2469−2473.

Espín, J. C.; García-Ruiz, P. A.; Tudela, J.; García-Cánovas, F. Study of stereospecificity in mushroom tyrosinase. *Biochem. J.* **1998d**, *331*, 547−551.

Espín, J. C.; García-Ruiz, P. A.; Tudela, J.; Varón, R.; García-Cánovas, F. Monophenolase and diphenolase reaction mechanisms of apple and pear polyphenol oxidases. *J. Agric. Food Chem.* **1998e**, *46*, 2968−2975.

Hammond, B. W.; Nichols, R. Carbohydrate metabolism in *Agaricus bisporus* (Lange) Sing.: changes in soluble carbohydrates during growth of mycelium and sporophore. *J. Gen. Microbiol.* **1976**, *93*, 309−320.

Jandel Scientific. *Sigma Plot 2.01 for Windows*; Jandel Scientific: Corte Madera, CA, 1994.

Kahn, V.; Zakin, V. Effect of kojic acid on the oxidation of trihydroxyphenols by mushroom tyrosinase. *J. Food Biochem.* **1995**, *18*, 427−433.

Kenten, R. H. Latent phenolase in extracts of broad-bean (*Vicia faba* L.) leaves 1. Activation by acid and alkali. *Biochem. J.* **1957**, *67*, 300−307.

Kermasha, S.; Goetghebeur, M.; Monfette, A.; Metche, M.; Rovel, B. Studies on inhibition of mushroom polyphenol oxidase using chlorogenic acid as substrate. *J. Agric. Food Chem.* **1993**, *41*, 526−531.

King, R. S.; Flurkey, W. H. Effects of limited proteolysis on broad bean polyphenoloxidase. *J. Sci. Food Agric.* **1987**, *41*, 231−240.

Martínez, M. V.; Whitaker, J. R. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **1995**, *6*, 195−200.

Moore, B. M.; Flurkey, W. H. Sodium dodecyl sulfate activation of a plant polyphenol oxidase. *J. Biol. Chem.* **1990**, *265*, 4982−4988.

Nellaiappan, K.; Sugumaran, M. On the presence of prophenoloxidase in the hemolymph of the horseshoe crab, *Limulus*. *Comp. Biochem. Physiol.* **1996**, *113B*, 163−168.

Prota, G. Progress in the chemistry of melanin and related metabolites. *Med. Res. Rev.* **1988**, *8*, 525−556.

Robinson, S. P.; Dry, I. B. Broad bean leaf polyphenol oxidase is a 60-Kilodalton protein susceptible to proteolytic cleavage. *Plant Physiol.* **1992**, *99*, 317−323.

Rodríguez-López, J. N.; Tudela, J.; Varón, R.; García-Carmona, F.; García-Cánovas, F. Analysis of a kinetic model for melanin biosynthesis pathway. *J. Biol. Chem.* **1992**, *267*, 3801−3810.

Ros, J. R.; Rodríguez-López, J. N.; García-Cánovas, F. Kinetics study of the oxidation of 4-*tert*-butylphenol by tyrosinase. *Eur. J. Biochem.* **1994a**, *222*, 449−452.

Ros, J. R.; Rodríguez-López, J. N.; García-Cánovas, F. Tyrosinase: kinetic analysis of the transient phase and the steady state. *Biochim. Biophys. Acta* **1994b**, *1204*, 33−42.

Soler-Rivas, C.; Arpin, N.; Olivier, J. M.; Wichers, H. J. Activation of tyrosinase in *Agaricus bisporus* strains following infection by *Pseudomonas tolaasii* or treatment with a tolaasin-containing preparation. *Mycol. Res.* **1997**, *101*, 375−382.

Sugumaran, M.; Nellaiappan, K. Lysolecithin-a potent activator of prophenoloxidase from the hemolymph of the lobster, *Homarus americanas*. *Biochem. Biophys. Res. Commun.* **1991**, *176*, 1371−1376.

Van Gelder, C. W. G.; Flurkey, W. H.; Wichers, H. J. Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry* **1997**, *45*, 1309−1323.

Van Leeuwen, J.; Wichers, H. Tyrosinase activity and isoform composition in separate tissues during development of *Agaricus bisporus* fruit bodies. *Mycol. Res.* **1999**, *103*, 413−418.

Waite, J. H. Calculating extinction coefficients for enzymatically produced *o*-quinones. *Anal. Biochem.* **1976**, *75*, 211−218.

Whitaker, J. R. Polyphenol oxidase. In *Food Enzymes Structure and Mechanism*; Wong, D. W. S., Ed.; Chapman Hall: New York, 1995; pp 271−307.

Wichers, H. J.; van den Bosch, T.; Gerritsen, Y. A.; Oyevaar, J. I.; Ebbelaar, M. C. E. M.; Recourt, K. Enzymology and molecular biology of *Agaricus bisporus* tyrosinase. In *Mushroom Science XIV, Science and Cultivation of Edible Fungi*; Elliot, T. J., Ed.; Balkema: Rotterdam, The Netherlands, 1995; Vol. 2, pp 723−728.

Yamaguchi, M.; Hwang, P. M.; Campbell, J. D. Latent *o*-diphenol oxidase in mushrooms (*Agaricus bisporus*). *Can. J. Biochem.* **1970**, *48*, 198−202.

Received for review December 7, 1998. Revised manuscript received June 9, 1999. Accepted June 10, 1999. J.C.E. is holder of Postdoctoral Grant FAIR/CT97-5004 from the European Commission under the framework of the Agriculture, Agro-Industry and Fisheries (FAIR) program.

JF981334Z