Purpling Reaction of Sinapic Acid Model Systems Containing L-DOPA and Mushroom Tyrosinase

To explain the formation of purple lesions in washed mushrooms during postharvest storage, the reactions of phenolic acids with quinones formed in the oxidation of catechol and L-DOPA by mushroom tyrosinase were investigated using a diode array UV–visible spectrophotometer. Among the phenolic acids tested, only gallic acid induced a purple color in the oxidation of catechol by tyrosinase, while gallic, ferulic, and sinapic acids induced a purple color in the oxidation of L-DOPA by tyrosinase. Sinapic acid was highly reactive with a melanogenic quinone derived from L-DOPA in producing a stable purple pigment with λ_{max} at 320 and 530 nm. This melanochrome analogue, ostensibly with the spectral properties of melanochrome (λ_{max} at 300 and 540 nm), was not produced in the γ-L-glutaminyl-4-hydroxybenzene tyrosinase–sinapic acid system. Isozymes of commercial and partially purified mushroom tyrosinase, separated by electrophoresis, were all active in inducing purpling in the L-DOPA–sinapic acid system. It is suggested that the formation of purple pigment may be due to the nonenzymatic polymerization of a canonical form of indole-5,6-quinone, derived from L-DOPA, with a mesomeric form of sinapic acid.

INTRODUCTION

During the storage of washed fresh mushrooms, a purple discoloration is sometimes seen on the pileus (cap) surface, often preceding the appearance of bacterial blotch lesions.

When L-DOPA is oxidized by mushroom tyrosinase, melanin pigments form through a series of oxidation–reduction reactions as follows: L-DOPA → dopaquinone → leucodopachrome → dopachrome → 5,6-dihydroxyindole → indole-5,6-quinone → melanochrome → melanin (Korner and Pawelek, 1980; Lerner and Fitzpatrick, 1950; Swan, 1974).

Among the various intermediates, melanochrome, a purple product, characterized by absorption maxima at 300 and 540 nm, which forms during the polymerization of indole-5,6-quinone to melanin, has been observed (Mason and Peterson, 1965). However, melanochrome has never been observed as a direct product of L-DOPA oxidation by tyrosinase. The existence of melanochrome as an intermediate was inferred from the action of tyrosinase on a 5,6-dihydroxyindole (Mason and Peterson, 1965).

In addition, some researchers found evidence for a purple reaction product during enzymatic oxidation of catechol, methylcatechol, and L-DOPA in the presence of external nucleophiles. Mason and Peterson (1955, 1965) showed that a purple color was produced in the reaction of *o*-benzoquinones (or 4-methyl-*o*-benzoquinone) derived from catechol (or methylcatechol) with secondary amino acids. They also showed that thiol-containing compounds react with melanogenic quinones (indole-5,6-quinone) derived from L-DOPA to produce a purple color. However, the latter reaction, which occurs when tyrosinase acts upon 5,6-dihydroxyindole, does not appear to be pertinent to mushroom because the dopaquinone of L-DOPA combines with free cysteine or with cysteinyl residues in glutathione or in protein to produce a colorless material, which inhibits the subsequent conversion of quinones to melanin (Graham et al., 1978; Sanada et al., 1972; Scheulen et al., 1975). Thus, the above reactions cannot account for the purple discoloration in mushroom. Until now, few fundamental studies on the physiology and biochemistry of purpling reactions in mushroom have been reported, in spite of extensive studies of chemical reactions involved in the process of melanization (Cabanes et al., 1987; Garcia-Canovas et al., 1982; Garcia-Carmona et al., 1982; Jimenez et al., 1984).

Phenolic acids, such as benzoic and cinnamic acid derivatives, are widely distributed throughout the plant kingdom. They play a central role in the biosynthesis of lignin, flavonoids, and related compounds (Harborne and Simmonds, 1964). These acids do not usually occur in the free state but rather as glycosides or as esters of glucose (Asen and Emsweller, 1962; Pearl and Darling, 1962). The cultivated mushroom (*Agaricus bisporus*) has the ability to break down a variety of phenolic compounds and to utilize the energy derived from this process (Cain et al., 1968; Paranjpe et al., 1978).

Recently we investigated the effect of various phenolic acids on the conversion of L-DOPA to melanin by mushroom tyrosinase and noted that sinapic acid reacts with a quinone derived from L-DOPA to produce a purple pigment with λ_{max} at 320 and 530 nm, which could not be detected in the process of melanization from L-DOPA.

This research utilized a model system containing L-DOPA, sinapic acid, and mushroom tyrosinase to develop a better understanding of the purple lesion which has been observed in washed mushroom during postharvest storage.

MATERIALS AND METHODS

Materials. Mushroom tyrosinase (EC 1.14.18.1; monophenol, dihydroxyphenylalanine:oxygen oxidoreductase), L-DOPA, pyrocatechol, and various phenolic acids except the three phenolic acids specified below were obtained from Sigma Chemical Co. (St. Louis, MO). γ-L-Glutaminyl-4-hydroxybenzene (GHB) was extracted from the gill tissue of mushroom following the procedure of Rast et al. (1979) and identified by UV and ^{1}H NMR spectroscopy (^{1}H NMR spectra were obtained with a JEOL GX-400 instrument with D_2O). Vanillic, gentisic, and sinapic acids as well as sodium periodate were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals used for this work were of analytical grade.

Methods. *Spectrophotometric Assay.* Oxidation of L-DOPA by mushroom tyrosinase was followed spectrophotometrically.

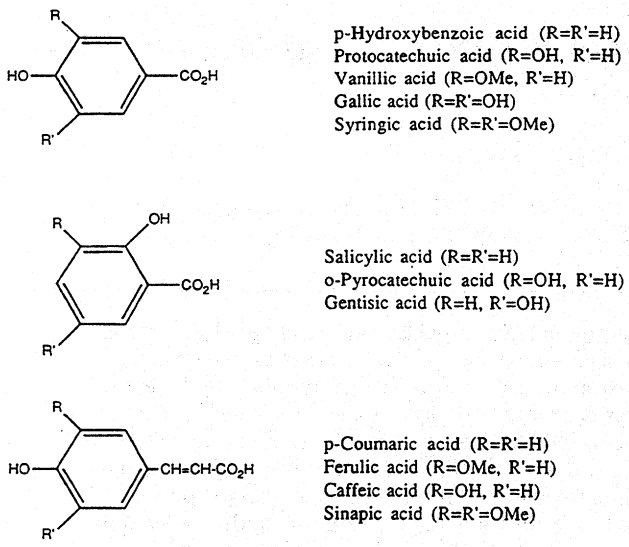


Figure 1. Structures of benzoic and cinnamic acid derivatives.

Reaction conditions are specified in the legends to the figures and tables. The spectrophotometric measurements were carried out at 25 °C in a Hewlett-Packard 8452A diode array spectrophotometer equipped with a thermostated cuvette holder and a recorder.

Partial Purification of Mushroom PPO. Mushroom PPO was prepared by a slight modification of the procedure reported by Ingebrigtsen et al. (1989). Fresh mushrooms were freeze-dried, ground to a fine powder in liquid nitrogen, and then blended with 20 volumes (w/v) of 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM ascorbic acid and 0.01% dithiothreitol and flushed with N_2 gas for 1 min. The homogenate was centrifuged at 12000*g* for 20 min at 4 °C. The supernatant was placed in a dialysis bag (Spectra/Por, MWCO 12 000–14 000, Spectrum Medical Industries, Inc., Houston, TX), concentrated to 2.0 mL with polyethylene glycol (PEG) 6000, and then recentrifuged at the same speed to obtain a partially purified PPO. This enzyme solution was subjected to electrophoresis.

Polyacrylamide Gel Electrophoresis. Native polyacrylamide gel electrophoresis (PAGE) was performed in Mini-Protean II ready gels (7 × 10 cm slab gels of 1.0-mm thickness, 7.5% single percentage gel, 0.375 M Tris-HCl, pH 8.8) (Bio-Rad Laboratory, Richmond, CA) according to the "Bio-Rad Laboratory Instruction Manual" (1989). Samples were made approximately 10% in glycerol before application. The electrophoretic run was at 4 °C and 100-V constant voltage. After electrophoresis, enzyme activity and purpling reaction bands were located by staining with 1 mM L-DOPA and 1 mM L-DOPA plus 0.1 mM sinapic acid, respectively.

RESULTS AND DISCUSSION

Spectroscopic Observations. The spectra that were observed during the reactions of various phenolic acids (Figure 1) with enzyme-oxidized L-DOPA and catechol are summarized in Table 1.

During the enzymatic oxidation of catechol without addition of phenolic acids, transitory absorption maxima developed at 390 nm, characteristic of *o*-benzoquinones. Poorly defined intermediate spectra developed during the subsequent polymerization of the quinones, and general absorption gradually appeared. In the presence of most of the phenolic acids, spectral changes were shown to be similar to those observed with tyrosinase-mediated catechol oxidation. However, when the same oxidation was carried out with gallic acid (Table 1B), new absorption maxima with λ_{max} at 270 and 563 nm appeared within the first minute of reaction as the reaction mixture became purple. Thereafter, a spectrum change *slowly* occurred in the visible region (as the sample turned from purple to black-green), and finally, a general absorption band developed. It is well-known that enzymatically generated quinones as well as those formed by coupled oxidation can react with one another or condense with hydroquinones (Gramshaw, 1970; McDonald and Hamilton, 1973) and polymerize (Cheynier et al., 1989; Cheynier and Ricardo, 1991). Therefore, purple pigments formed during enzymatic oxidation of catechol in the presence of gallic acid may be due to polymerization and condensation of quinones (formed by enzymatic or coupled oxidation of catechol or gallic acid) with hydroquinones of the parent phenolic acids.

The spectroscopic changes that occur during the enzymatic oxidation of L-DOPA have already been established (Mason and Peterson, 1965). They consist of shifts from L-DOPA to dopachrome, and then to melanochrome, and finally to melanin. Of these, only the dopachrome chromophore (λ_{max} at 302 and 475 nm) could be detected by rapid scanning spectroscopy (Graham and Jeffs, 1977). This spectroscopic sequence was nearly unaltered in the presence of most phenolic acids. However, in the presence of gallic acid, a new spectrum developed within the first

Table 1. Spectra of Reaction Products Arising from the Enzymatic Oxidation of L-DOPA and Catechol in the Presence of Various Phenolic Acids[a]

phenolic acid	absorption maxima[b] (nm) catechol	L-DOPA
A. none	390 (A)	305, 475 (A)
	250 (360, 450, 520) (B)	300, 540 (B)
	general absorption (C)	general (C)
B. benzoic acid derivatives		
1. *p*-hydroxybenzoic acid	390 (A)	300 (s), 480 (A)
	general (B)	general (B)
2. protocatechuic acid	390 (A)	300 (s), 480 (A)
	general (B)	general (B)
3. vanillic acid	250, 280, 390 (A)	480 (A)
	general (B)	general (B)
4. gallic acid	270, 563 (A)	562 (A)
	370 (s), 558 (B)	497 (B)
	540 (s) (C)	480 (C)
5. syringic acid	260, 390 (A)	480 (A)
	general (B)	general (B)
6. gentisic acid	320, 390 (s) (A)	480 (A)
	400 (s), 500 (s) (B)	general (B)
	480 (s) (C)	
7. salicylic acid	390 (A)	480 (A)
	general (B)	general (B)
8. *o*-pyrocatechuic acid	320, 390 (s) (A)	480 (A)
	398 (s), 500 (s) (B)	general (B)
	general (C)	
C. cinnamic acid derivatives		
9. *trans*-cinnamic acid	390 (A)	480 (A)
	general (B)	490 (s) (B)
10. *p*-coumaric acid	390 (A)	480 (A)
	general (B)	general (B)
11. *o*-coumaric acid	260, 320, 390 (A)	480 (A)
	general (B)	general (B)
12. *m*-coumaric acid	390 (A)	480 (A)
	general (B)	general (B)
13. ferulic acid	450 (A)	300, 475 (A)
	420 (s) (B)	490 (B)
	general (C)	310, 530 (C)
14. caffeic acid	310, 390 (A)	480 (A)
	general (B)	general (B)
15. sinapic acid	270, 310, 480 (A)	300, 480 (A)
	420 (s) (B)	518 (B)
	420 (s) (C)	315, 537 (C)

[a] The reaction mixture included, in a total volume of 3 mL, 0.3 mM L-DOPA, 50 mM sodium phosphate buffer (pH 6.5), 4 μg of mushroom tyrosinase, and 0.1 mM various phenolic acids. [b] Absorption maxima and shoulder(s) appearing in successive absorption spectra observed following enzyme addition and disappearance of substrate's absorption spectrum: (A) immediately after, (B) after 15–30 min, (C) after 1–12 h.

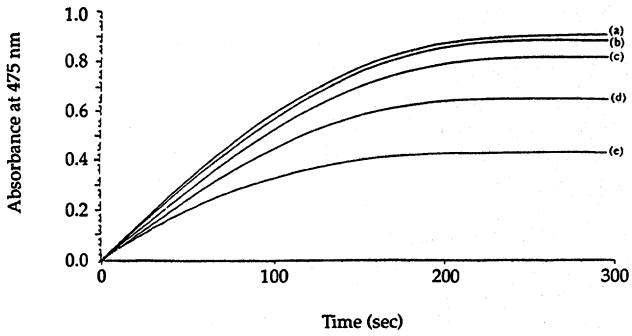


Figure 2. Effect of different concentrations of sinapic acid on the L-DOPA oxidase activity of mushroom tyrosinase. The reaction mixture included, in a total volume of 3 mL, 0.3 mM L-DOPA, 50 mM sodium phosphate buffer (pH 6.5), 4 μg of mushroom tyrosinase (added last), and sinapic acid (in mM) as follows: a, none; b, 0.033; c, 0.33; d, 0.83; e, 1.66.

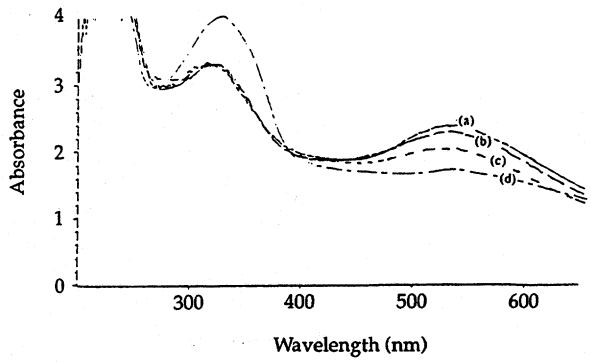


Figure 3. UV–visible spectra of reaction products obtained from different enzymatic reactions: (a) reaction when 1.0 mM sinapic acid was added to reaction mixture after 0.5 mM L-DOPA was reacted with 4 μg of tyrosinase for 2 min; (b) reaction when 0.5 mM L-DOPA and 1.0 mM sinapic acid were oxidized together by 4 μg of tyrosinase for 12 h; (c) reaction when 0.5 mM L-DOPA was added to reaction mixture after 1.0 mM sinapic acid was reacted with 4 μg of tyrosinase for 2 min; (d) reaction when 0.5 mM L-DOPA was added to reaction mixture after 1.0 mM sinapic acid was reacted with 4 μg of tyrosinase for 4 h. The spectrum was scanned after 12 h of reaction against a blank containing tyrosinase and buffer.

minute, with λ_{max} at 562 nm (Table 1B) corresponding to the appearance of a purple color. During incubation, the maximum absorption band at 562 nm shifted *rapidly* to a lower wavelength, and finally, a band with an intense red-orange color developed at 480 nm. Thus, in the presence of gallic acid, the oxidation of L-DOPA and catechol by tyrosinase produced a similar purple pigment, although the purple color in L-DOPA oxidation disappeared *rapidly* compared to that in catechol oxidation.

When the L-DOPA oxidation was carried out in the presence of ferulic or sinapic acid, new absorption spectra with λ_{max} at 310 nm and 530 nm (ferulic acid) or at 315 and 537 nm (sinapic acid), apparently characteristic of melanochrome, slowly developed over 12 h through subsequent polymerization after the initial appearance of the dopachrome spectrum (Table 1C). The purple intermediate, formed by the model system of L-DOPA–tyrosinase–sinapic acid, was highly stable at ambient temperature and retained its color for about 2 days, while that formed from ferulic acid was labile and quickly proceeded to form melanin.

Fungi are well-known for their ability to break down and utilize phenolic compounds as a source of energy. Paranjpe et al. (1978) identified sinapic and ferulic acids in mushroom by means of paper and gas–liquid chromatography. Therefore, traces of sinapic and ferulic acids, which may be generated during breakdown of phenolic compounds by microorganisms or in hyphae, might initiate the synthesis of purple pigments in the presence of L-DOPA and tyrosinase. Thus, this phenomenon may explain the formation of purple lesions in mushroom at a relatively late stage of storage. Therefore, we investigated in some detail the chemical reactions involved in purpling induced by sinapic acid during the enzymatic oxidation of L-DOPA.

Effect of Sinapic Acid on the L-DOPA Oxidase Activity of Mushroom Tyrosinase. As shown in Figure 2, sinapic acid inhibited slightly the enzymatic oxidation of L-DOPA at lower concentration (less than 0.33 mM), while it showed a moderate inhibitory effect at higher concentration (greater than 0.33 mM). Aromatic carboxylic acids and some of their derivatives are known potent inhibitors of tyrosinase (Gunata et al., 1987; Martinez-Cayuela et al., 1988). Pifferi et al. (1974) studied the inhibition of sweet cherry fruit PPO by cinnamic acid derivatives, and they demonstrated that the inhibitory strength of the cinnamic acids was due to the presence of a double bond conjugated with the carboxyl group and the benzene nucleus, which showed a steric interference acting on the active site of the enzyme. Gunata et al. (1987) have observed that di (meta) methylation and esterification of cinnamic acids decrease the strength of their inhibitory effect on grape PPO. These results suggested that sinapic acid did not act as a substrate but rather as an inhibitor for mushroom tyrosinase.

Oxidation of L-DOPA by Tyrosinase in the Presence of Sinapic Acid. To establish the best model system to study purpling reactions, we first investigated four different enzymatic reaction sequences and the effect of sinapic acid concentration on purple pigment formation before chemical reactions involved in purple pigment formation were studied in detail.

The spectra of purple pigments formed from the different enzymatic reactions are presented in Figure 3. Among four sequences tested, addition of sinapic acid after reaction of L-DOPA with tyrosinase for 2 min (a) gave the best yield of purple pigment, producing levels of dopachrome sufficient to react with sinapic acid. Dopachrome catalyzed the formation of material absorbing at 530 nm (purple pigment) by means of nonenzymatic reaction with sinapic acid. Sequence b, simultaneous reaction of sinapic acid and L-DOPA with tyrosinase for 12 h, would then represent a small decrease in the formation of purple pigment at 530 nm due to the inhibitory effect of sinapic acid on enzymatic oxidation of L-DOPA. Sequence c, addition of L-DOPA after reaction of sinapic acid with tyrosinase for 2 min, would represent the inhibition of purple pigment formation by means of steric hindrance in the interaction of sinapic acid with enzyme. The addition of L-DOPA after reaction of sinapic acid with tyrosinase for 4 h (d) did not yield a purple pigment, since sinapic acid quinone formed in the enzymatic oxidation of sinapic acid inactivated the enzyme, thereby preventing the conversion of L-DOPA to dopachrome. This inactivation may occur by binding of quinone on the active site of the enzyme or by a free-radical mechanism as described previously by some authors (Golan-Goldhirsh and Whitaker, 1985; Wood and Ingraham, 1965). In view of these results, for the formation of purple pigments to occur, the enzymatic oxidation of L-DOPA to dopachrome should take place first, followed by nonenzymatic polymerization between the quinone derived from L-DOPA and sinapic acid. When L-DOPA solution is applied to the surface of a mushroom, an orange-red color develops within a few seconds. This color slowly turns purple when

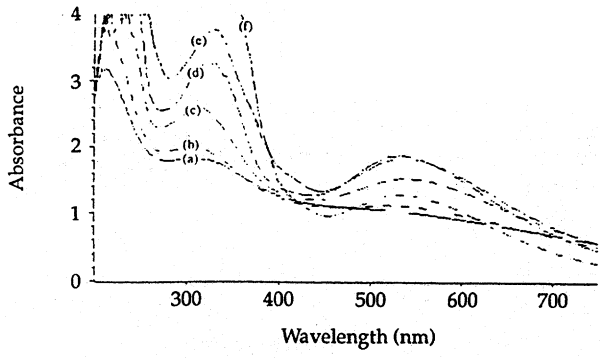


Figure 4. Effect of different concentrations of sinapic acid on the purple pigments obtained when L-DOPA is oxidized by mushroom tyrosinase in the presence of sinapic acid. The reaction mixture included, in a total volume of 3 mL, 0.3 mM L-DOPA, 50 mM sodium phosphate buffer (pH 6.5), 4 μg of mushroom tyrosinase (added last), and sinapic acid (in mM) as follows: a, none; b, 0.033; c, 0.1; d, 0.33; e, 0.66; f, 1.66. The spectrum was scanned after 12 h of reaction against a blank containing tyrosinase and buffer.

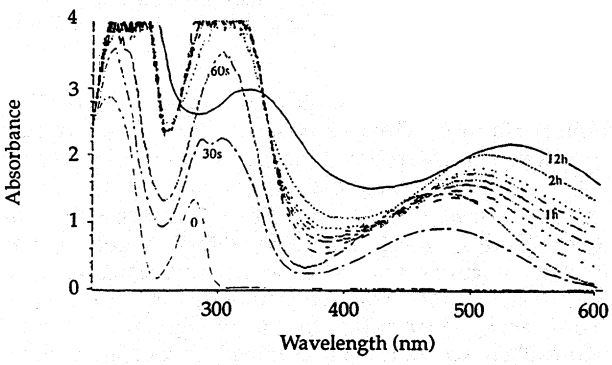
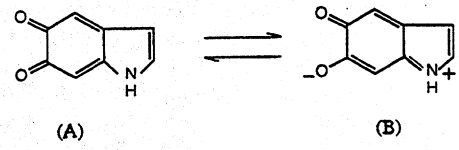


Figure 5. Spectral changes occurring during the oxidation of L-DOPA by tyrosinase in the presence of sinapic acid. The reaction mixture included, in a total volume of 3 mL, 0.3 mM L-DOPA, 50 mM sodium phosphate buffer (pH 6.5), 4 μg of mushroom tyrosinase, and 0.33 mM sinapic acid (added after 1 min of enzymatic oxidation of L-DOPA). The spectrum was scanned at the indicated times against a blank containing tyrosinase and buffer.

the solution is absorbed by mushroom tissue. This is consistent with the fact that L-DOPA oxidation precedes reaction with sinapic acid.

The data in Figure 4 illustrate that the formation of purple products with λ_{max} at 320 and 530 nm is stimulated by increasing the concentration of sinapic acid (to 0.66 mM), even though this would slightly inhibit the rate of L-DOPA oxidation, monitored at 475 nm (Figure 2). However, purpling decreased at the highest sinapic acid concentration (1.66 mM). Thus, it is possible to control the extent of purpling by varying the sinapic acid concentration.

The purpling reaction can be observed in greater detail in the absorption spectra of pigments formed when L-DOPA was oxidized with tyrosinase at pH 6.5 in the presence of sinapic acid at 25 °C (Figure 5). As expected, dopachrome, with λ_{max} at 302 and 475 nm, developed completely within 1 min after initiation of the reaction. Thereafter, addition of 0.3 mM L-DOPA to the reaction mixture did not result in a further increase in dopachrome absorption (data not shown). With the addition of sinapic acid, the peak shifted slowly from 475 to 500 nm over the course of 2 h and then to 530 nm over an additional 10 h as polymerization occurred. A purple intermediate with λ_{max} at 320 and 530 nm, apparently characteristic of melanochrome, developed during incubation, although the UV absorption band showed a shift to a higher wavelength than that of melanochrome, indicating that different proportions of side-chain conjugation may be formed in the course of nonenzymatic oxidative polymerization. In contrast, when L-DOPA was oxidized with mushroom tyrosinase without the addition of sinapic acid, neither the 320 and 530 nm peaks, attributable to purple pigments, nor crossing of successive tracings was observed. Only the general absorption characteristic of melanin developed during the same period. Thus, the formation of purple pigments was due to nonenzymatic polymerization of dopachrome with sinapic acid. However, as suggested by Mason and Peterson (1965) for enzymatic oxidation of 5,6-dihydroxyindole, the initial dopachrome formed can be transferred rapidly by addition of either water or sinapic acid to indole-5,6-quinone. In addition, when this same oxidation was carried out in water containing 30% ethanol, a purple color formed quickly without a long polymerization step (data not shown), indicating that canonical forms **A** and **B**, or protonated or unprotonated states, of



indole-5,6-quinone may exist as transitory intermediates, as observed earlier with 2-carboxyindole-5,6-quinone (Mason and Peterson, 1965). Therefore, it seems likely that indole-5,6-quinone instead of dopachrome may polymerize nonenzymatically with sinapic acid to give a purple pigment.

Identification of the purple pigments thus obtained should provide complementary information on the mechanisms responsible for their formation. Some instrumental analyses including IR, NMR, and mass spectrometry are underway to achieve structural characterization. However, this has proved very difficult so far because of the high reactivity of these intermediates and fast interconversion during storage. Similarly, it has not yet been possible to isolate, purify, and characterize the purple pigment produced in washed mushrooms because of its reactivity.

Oxidation of GHB by Tyrosinase in the Presence of Sinapic Acid. GHB, the most predominant phenolic substrate of tyrosinase in mushroom sporophores, is well-known to have important roles in tissue browning and melanin formation, as well as in the induction of spore dormancy in *A. bisporus* (Oka et al., 1981; Rast et al., 1979; Stussi and Rast, 1981; Vogel and Weaver, 1972). GHB is known to be hydroxylated by tyrosinase to γ-L-glutaminyl-3,4-dihydroxybenzene (GDHB), which is then oxidized to γ-L-glutaminylbenzoquinone (GBQ). The latter then undergoes a base-catalyzed nonenzymatic transformation to 2-hydroxy-4-iminoquinone, which finally leads to the formation of polymerized melanin-like compounds (Boekelheide et al., 1979, 1980).

The enzymatic oxidation of GHB in the presence of sinapic acid was also investigated to differentiate between reactions of L-DOPA and GHB. As can be seen in Figure 6, upon oxidation of GHB by tyrosinase, GBQ with absorption maxima at 285 and 460 nm developed within 10 min (this peak shifts toward λ_{max} at 490 nm during 1 h of reaction). Once formed, GBQ is unstable in solution and can potentially undergo a number of degradative and polymerization reactions. When sinapic acid was added to the reaction mixture after 10 min and the reaction was allowed to proceed for 5 min, the original absorption spectrum of GBQ disappeared almost instantly, followed

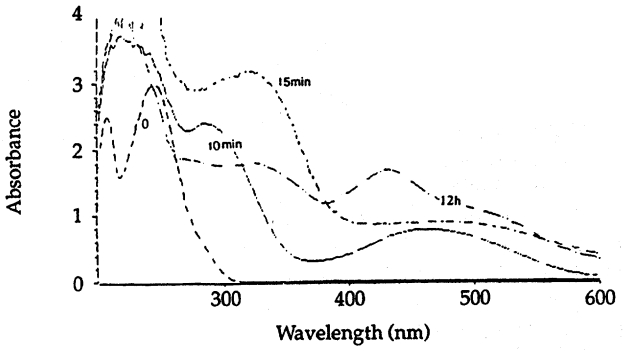


Figure 6. Spectral changes occurring during the oxidation of GHB by tyrosinase in the presence of sinapic acid. The reaction mixture included, in a total volume of 3 mL, 0.18 mM GHB, 50 mM sodium phosphate buffer (pH 6.5), 4 μg of mushroom tyrosinase, and 0.33 mM sinapic acid (added after 10 min of enzymatic oxidation of GHB). The spectrum was scanned at the indicated times against a blank containing tyrosinase and buffer.

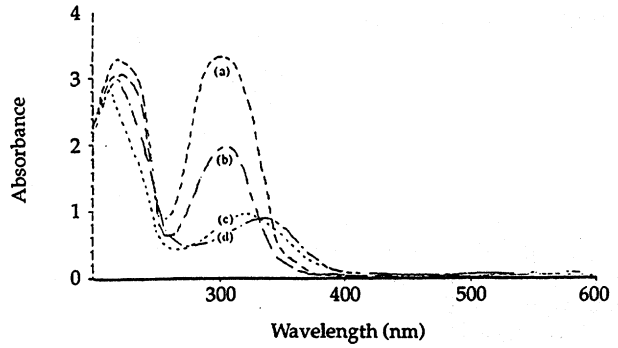


Figure 7. Oxidation of sinapic acid by sodium periodate at 25 °C in 50 mM sodium phosphate buffer (pH 6.5): (a) sinapic acid 0.25 mM; (b) sinapic acid (0.25 mM) was reacted with an equimolar ratio of $NaIO_4$ (0.25 mM), recorded at 3 s from the beginning of the reaction; (c) recorded at 5 min from the beginning of the reaction, conditions as expressed in (b); (d) sinapic acid (0.25 mM) was reacted with 0.5 mM $NaIO_4$, recorded at 5 min from the beginning of the reaction.

by the appearance of a single maximum at 320 nm, indicating that the addition of sinapic acid to GBQ and the nonenzymatic base-catalyzed cleavage of GBQ took place simultaneously. Over 12 h, the reaction produced an intense red color with absorption maxima at 330 (shoulder) and 430 nm. Thus, the enzymatic oxidation of GHB in the presence of sinapic acid resulted in the formation of a red color instead of a purple color, which we previously observed during the enzymatic oxidation of L-DOPA with sinapic acid (Figure 5). Although hydroxylation, oxidation, and polymerization of GHB would follow the same course as that of tyrosine (Boekelheide et al., 1979, 1980), this pathway is distinctly different in that GBQ undergoes a nonenzymatic base-catalyzed cleavage to yield 2-hydroxy-4-iminoquinone, without a further ring substitution reaction (cyclization following a Michael intramolecular 1,4-addition), which occurs during the conversion of dopaquinone to dopachrome. Consequently, the oxidation of GHB by tyrosinase in the presence of sinapic acid does not contribute to a purpling reaction in mushroom but rather to browning.

Oxidation of Sinapic Acid by Tyrosinase and Sodium Periodate. To know the effect of sinapic acid on polymerization, we compared the oxidation of sinapic acid by sodium periodate ($NaIO_4$) and by tyrosinase.

When sinapic acid was incubated with an equimolar ratio of sodium periodate (Figure 7), there was a progressive decline in absorbance at 300 nm, with a shift of the λ_{max}

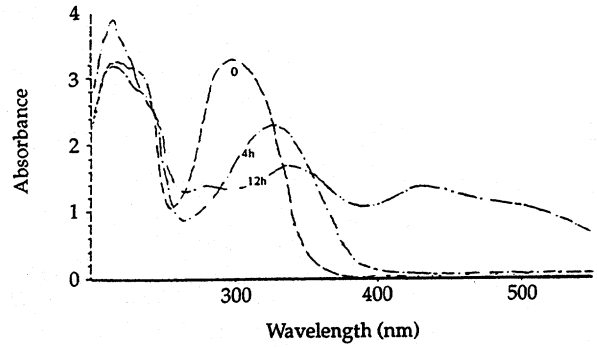
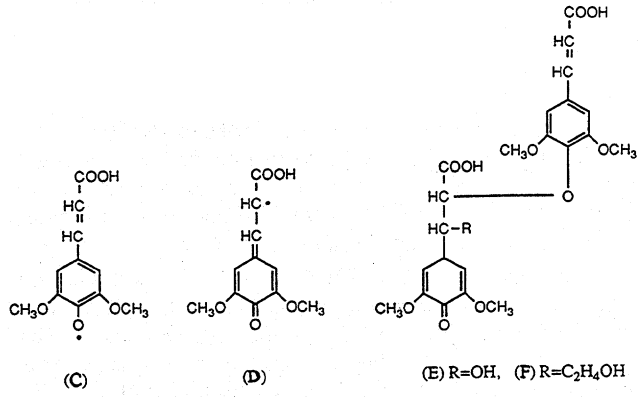


Figure 8. Spectral changes occurring during enzymatic oxidation of sinapic acid. The reaction mixture included, in a total volume of 3 mL, 0.33 mM sinapic acid, 50 mM sodium phosphate buffer (pH 6.5), and 4 μg of mushroom tyrosinase. The spectrum was scanned at the indicated times against a blank containing tyrosinase and buffer.

to 320 nm. Furthermore, with addition of a 2-fold excess of periodate to the reaction mixture, the peak at 320 nm shifted to 340 nm. Thereafter, the peak at 340 nm disappeared, and finally, the reaction mixture turned brown after 12 h of reaction.

In contrast, when sinapic acid was oxidized by tyrosinase at pH 6.5 (Figure 8), an initial maximum at 300 nm shifted slowly to 330 nm (light brown product), accompanied by a decrease in absorbance after 4 h of initial reaction. Thereafter, the light brown intermediate slowly polymerized, and finally a red-orange product developed with absorption maxima at 340 and 430 nm and a shoulder at 280 nm after 12 h. This product did not appear in the chemical oxidation of sinapic acid by periodate. We suggest that enzymatic attack on sinapic acid causes the removal of the phenolic hydrogen atom, leaving a radical which can exist in two mesomeric forms (**C** and **D**). These



radicals are then dimerized. Furthermore, **C** and **D** can combine to form a quinone methide derivative (**E**) or to yield **F** with addition of a large amount of water by a free-radical mechanism of polymerization (Freudenberg, 1959). On the other hand, in the case of chemical oxidation of sinapic acid with an equimolar ratio of periodate, the quinone or hydroperoxide derivatives may be formed in place of mesomeric forms **C** and **D**. Further oxidation resulted in cleavage of the benzene ring and double bond to produce an intense brown color.

This result suggests that the mesomeric forms of sinapic acid as well as the semiquinone property of indole-5,6-quinone may more accurately account for the formation of purple pigments. This result also is helpful in explaining the difference between purpling reactions formed enzymatically and nonenzymatically (by $NaIO_4$ oxidation).

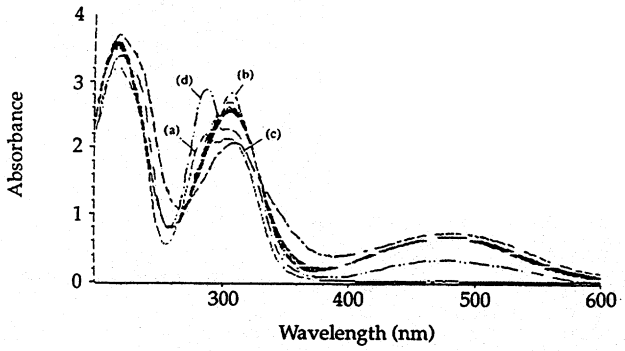


Figure 9. Oxidation of L-DOPA by sodium periodate at 25 °C in 50 mM sodium phosphate buffer (pH 6.5) in the presence of 0.25 mM sinapic acid; (a) L-DOPA (0.5 mM) plus sinapic acid; (b) L-DOPA (0.5 mM) was oxidized with an equimolar ratio of $NaIO_4$; (c) L-DOPA (0.5 mM) was oxidized with greater than 1 equiv of $NaIO_4$ (2.0 mM); (d) L-DOPA (2.0 mM) was oxidized with less than 1 equiv of $NaIO_4$ (0.5 mM). The spectrum was scanned after 5 min of reaction against a blank containing tyrosinase and buffer.

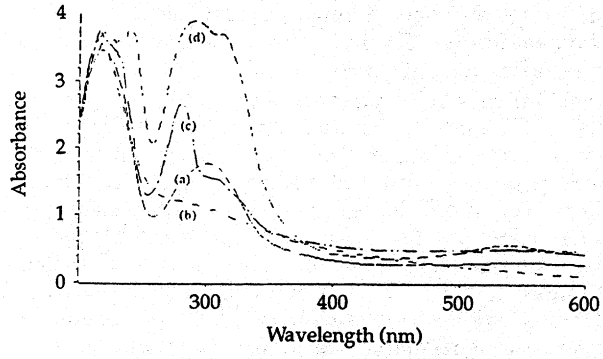


Figure 10. UV–visible spectra of reaction products formed in the oxidation of L-DOPA by periodate in the presence of sinapic acid: (a) reaction (b) in Figure 9; (b) reaction (c) in Figure 9; (c) reaction (d) in Figure 9; (d) L-DOPA (2.0 mM) and sinapic acid (1.0 mM) were oxidized with less than 1 equiv of $NaIO_4$ (0.5 mM). The spectrum was scanned after 12 h of reaction against a blank containing tyrosinase and buffer.

Oxidation of L-DOPA by Periodate in the Presence of Sinapic Acid. It is well established that the chemical oxidation of L-DOPA by sodium periodate leads to the production of dopaquinone and dopachrome (Garcia-Canovas, 1982; Graham and Jeffs, 1977).

When oxidation of L-DOPA was carried out with an equimolar ratio of periodate ([$NaIO_4$]/[L-DOPA] = 1.0) at pH 6.5 in the presence of sinapic acid (Figure 9b) the oxidation of L-DOPA to dopachrome occurred quickly with appearance of absorption maxima at 300 and 475 nm; periodate can also oxidize sinapic acid, so that no isosbestic point was detected. The spectrum of dopachrome was unchanged with increasing reaction time. After 12 h of incubation at 25 °C, the reaction mixture turned black-brown (Figure 10a).

When chemical oxidation by an excess sodium periodate ([$NaIO_4$]/[L-DOPA] = 4) was performed in the presence of sinapic acid (Figure 9c), the oxidation of L-DOPA and sinapic acid took place simultaneously. The absorbance at 300 nm decreased with a λ_{max} shift to 305 nm, while the absorption in the range 400–600 nm increased. This result suggested that the reaction is nearly instantaneous up to L-DOPA depletion, with an initial maximum at 300 nm which decreases and shifts as a result of addition of sinapic acid to the quinone ring. After 12 h of incubation at 25 °C, the reaction mixture turned brown-black (Figure 10b).

On the other hand, when chemical oxidation was carried out with less than 1 equiv of periodate ([$NaIO_4$]/[L-DOPA] = $^1/_4$) at pH 6.5 (Figure 9d), the development of dopachrome chromophore at 475 nm was slight, and the shift and diminution of this spectrum, attributable to the formation of adducts of dopachrome with sinapic acid, were not observed. This spectrum remains unchanged with increasing reaction time, as in the previous situation (Figure 9b). After 12 h of incubation at 25 °C, the reaction mixture turned slightly purple (Figure 10c). However, when the same oxidation was carried out with increasing sinapic acid concentration (1 mM), a purple pigment with indistinct absorption maxima at 280, 320, and 540 nm (weak shoulder) developed after 12 h (Figure 10d), indicating that L-DOPA in the reaction mixture was only partly oxidized with periodate, and then dopachrome reacted with sinapic acid to produce a purple color. Although a purple color was induced in the chemical oxidation of L-DOPA in the presence of sinapic acid, the absorption maxima of this purple pigment are *different* from those (320 and 530 nm) obtained in the enzymatic oxidation of L-DOPA with sinapic acid, which suggested that a specific side-chain conjugation between a canonical form derived from indole-5,6-quinone and a mesomeric form of sinapic acid may occur much better in the enzymatic oxidation of L-DOPA than in the nonenzymatic oxidation.

Taking into account these studies, purple pigments formed by sinapic acid in the course of the enzymatic oxidation of L-DOPA may be due to the polymerization and condensation of indole-5,6-quinone with radical intermediates (mesomeric forms, etc.) of sinapic acid, formed by reaction of sinapic acid with some quinones of L-DOPA. Chemical oxidation of L-DOPA and/or sinapic acid, as might result from the presence of hypochlorite in water used to wash mushrooms, is not likely to be a source of purpling. However, further study of the mechanisms involved in purpling reactions is needed to clarify these reactions.

Polyacrylamide Gel Electrophoresis. To demonstrate the reactivity of all isozymes of PPO in the purpling reaction, commercial tyrosinase and partially purified mushroom PPO were subjected to native electrophoresis, followed by isozyme localization using 1 mM L-DOPA and 1 mM L-DOPA plus 0.1 mM sinapic acid to induce browning and purpling, respectively. Four isozyme bands with similar mobility and intensity, which exhibit both L-DOPA oxidase activity and purpling reactions, were isolated from the two sources of PPO. The main band, with Rm 0.38, showed a strong intense purple color, which was retained for 12 h; the purple color of the other three bands disappeared slowly. Thus, we can detect electrophoretically the purpling reaction with all isozymes of mushroom PPO.

Conclusions. The purple discoloration that can occur during storage of washed mushrooms and may be induced by application of L-DOPA to the pileus surface is attributed to the reaction of a canonical form of indole-5,6-quinone with a radical mesomeric product of sinapic acid oxidation. The former is derived from L-DOPA via tyrosinase oxidation, while the latter reactant may be produced by the reaction of sinapic acid with quinones derived from L-DOPA. Other enzymatic and nonenzymatic reactions of phenolic compounds found in mushrooms that yielded purple products were not consistent with kinetic and spectral characteristics of mushroom purpling.

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