JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

REVIEWS

Mushroom Tyrosinase: Recent Prospects

SUNG-YUM SEO,* VINAY K. SHARMA, AND NITI SHARMA

Applied Biotechnology Laboratory, Department of Biology, College of Natural Sciences, Kongju National University, Kongju 314-701, South Korea

Tyrosinase, also known as polyphenol oxidase, is a copper-containing enzyme, which is widely distributed in microorganisms, animals, and plants. Nowadays mushroom tyrosinase has become popular because it is readily available and useful in a number of applications. This work presents a study on the importance of tyrosinase, especially that derived from mushroom, and describes its biochemical character and inhibition and activation by the various chemicals obtained from natural and synthetic origins with its clinical and industrial importance in the recent prospects.

Keywords: Mushroom tyrosinase; biochemical character; inhibitors; activators; clinical studies; immobilization

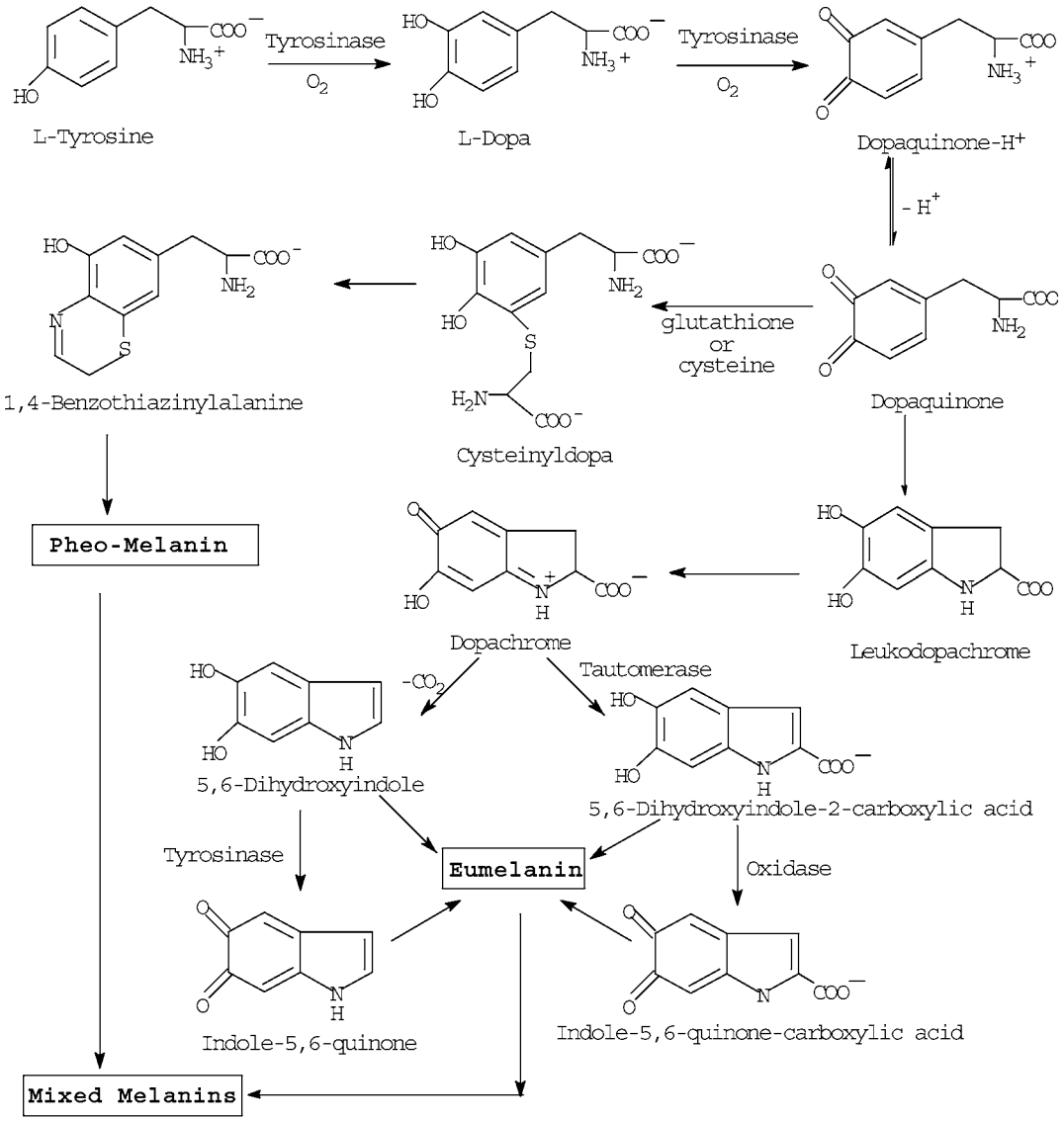
1. INTRODUCTION

Mushrooms have been consumed by humans since ancient times not only as a part of the normal diet but also as a delicacy due to their desirable taste and aroma. The use of mushrooms with therapeutic properties is growing day by day due to the range of side effects caused by conventional medicines. Among natural products, mushrooms have been recognized as potent candidates in clinical studies because they are readily obtained in relatively large quantities and are inexpensive. Over the past 30 years the enzyme tyrosinase (polyphenol oxidase, EC 1.14.18.1) has received considerable attention as an indispensable tool in the performance of studies on a wide range of topics. Since the first biochemical investigations were carried out in 1895 on the mushroom *Russula nigricans*, the cut flesh of which turned red and then black on exposure to air (*1*), a number of studies have been made to find the culprit mainly responsible for the color change, which is widely distributed through the phylogenetic scale from lower to higher life forms (*2*−*6*), although, in some cases, it is not detectable due to endogenous inhibitors (*7*, *8*). This enzyme was later identified as tyrosinase, the active site of which contains a binuclear copper cluster in the common mushroom (*Agaricus bisporus*) and in human malignant melanoma tyrosinase (*9*, *10*). In higher plants and fungi, tyrosinases occur in various isoforms such as immature, mature latent (*11*, *12*) and active forms; however, the biochemical description regarding the kinetic characterization and relationship between these isoforms is yet to be established. The biosynthetic pathway for melanin formation, operating in insects, animals, and plants, has largely been elucidated by Raper (*13*), Mason (*14*), and Lerner et al. (*15*). The first two steps in the pathway are the hydroxylation of monophenol to *o*-diphenol (monophenolase or cresolase activity) and the oxidation of diphenol to *o*-quinones (diphenolase or catecholase activity), both using molecular oxygen followed by a series of nonenzymatic steps resulting in the formation of melanin (*13*, *16*, *17*) as shown in **Scheme 1**, which plays a crucial protective role against skin photocarcinogenesis. The production of abnormal melanin pigmentation (melasma, freckles, ephelide, senile lentigines, etc.) is a serious esthetic problem in human beings (*18*). In fungi, the role of melanin is correlated with the differentiation of reproductive organs and spore formation, virulence of pathogenic fungi, and tissue protection after injury (*19*−*21*). In addition, tyrosinase is responsible for the undesired enzymatic browning of fruits and vegetables (*22*) that takes place during senescence or damage at the time of postharvest handling, which makes the identification of novel tyrosinase inhibitors extremely important. However, besides this role in undesired browning, the activity of tyrosinase is needed in other cases (raisins, cocoa, fermented tea leaves) where it produces distinct organoleptic properties. Mushroom tyrosinase is popular among researchers as it is commercially available and inexpensive and also there are easy tools to investigate the feature of this enzyme. Among mushrooms *A. bisporus* is the most commonly consumed species worldwide, and also it is a representative of its family; for this reason most of the research work is being carried out on this particular species. Moreover, all of the tyrosinases obtained from various species of mushroom have similar properties, so most of the studies reported in the present paper are related to *A. bisporus*. However, it is inevitable that we will be selective in our coverage, but an attempt will be made to deal with various aspects of mushroom tyrosinase including its biochemical characteristics, inhibitors, and activators from

* Author to whom correspondence should be addressed (telephone 82-41-850-8503; fax 82-41-854-8503; e-mail dnalove@kongju.ac.kr).

10.1021/jf020826f CCC: $25.00
Published on Web 04/12/2003

Scheme 1. Pathway of Melanogenesis (*13*, *16*, *17*)



various sources and its clinical and industrial importance in recent prospects.

2. BIOCHEMICAL CHARACTERISTICS OF MUSHROOM TYROSINASE

A number of research papers and reviews (*4*, *6*, *23*) have already been published on the structural and kinetic aspects of the enzyme tyrosinase; therefore, under this section we will briefly discuss its biochemical character in relation to domain structure, reaction mechanism, and substrate specificity with special reference to *A. bisporus*.

2.1. Domain Structure. Tyrosinase from *A. bisporus* was reported to be a heterotetramer comprising two heavy (H) and light (L) chains with a molecular mass of 120 kDa (*24*). Later, Robb and Gutteridge (*25*) identified two types of heavy chains H^{α} and H^{β} for the isozymes, $H_2{}^{\alpha}L_2$ and $H_2{}^{\beta}L_2$, corresponding to α and β, respectively. The two monomeric isoforms with both catecholase and cresolase activities were isolated from the fruit bodies of the mushroom (*26*) and occur as monomeric single-chain polypeptides with a molecular mass of 47 kDa under native conditions. The complete sequence of a tyrosinase clone for *A. bisporus* has been established by Wichers et al. (*27*); it showed a high degree of similarity with mushroom tyrosinase sequence. Basically, the enzyme tyrosinase has three domains, of which the central domain contains two Cu binding sites, called Cu_A and Cu_B. Several conserved sequences are found to be present in tyrosinases from different sources as shown in **Figure 1**. In fact, when all tyrosinase sequences were compared, the only conserved domain seems to be the central copper-binding domain, which also shares sequence homology with hemocyanins (Hcs), copper-containing oxygen carriers from the hemolymph of many molluscs and arthropods (*28*). Six conserved histidine residues (*29*) bind a pair of copper ions in the active site of the enzyme tyrosinase, which interact with both molecular oxygen **(Figure 2)** and its phenolic substrate. The location of cysteine (Cys) also plays an important role in the formation of disulfide linkages, which stabilize protein structure. The number of Cys residues varies from one organism to another, as along the N-terminal and central part of the protein, human and mouse tyrosinases have 17 Cys residues and plants have 11, whereas the C-terminal domain contains 1 Cys residue. Interestingly, *A. bisporus*, *Neurospora crassa*, and prokaryotic tyrosinase contain 0 or 1 Cys in mature protein. In mushroom tyrosinase sequence only 2 Cys residues are reported in the C-terminal domain (*6*). Various inhibitors and activators modulate the enzyme activity by binding at this site. To

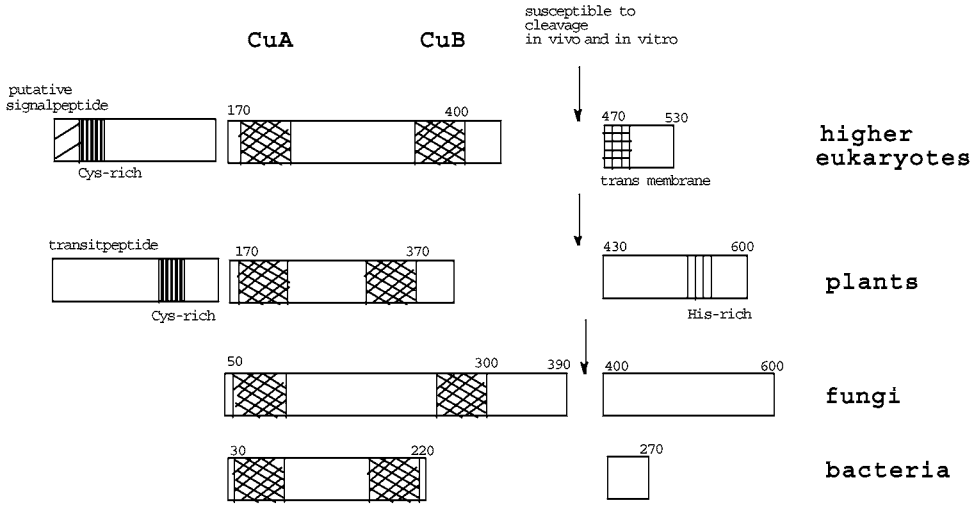


Figure 1. Domain structure of tyrosinases from different groups. Conserved Cu binding sites (CuA and CuB) are represented by cross-hatched areas along with some other sequences. Arrows indicate susceptible cleavage site in the enzyme (*6*).

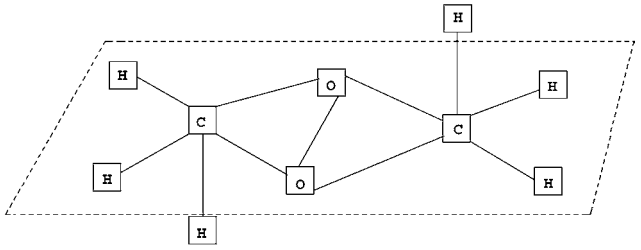
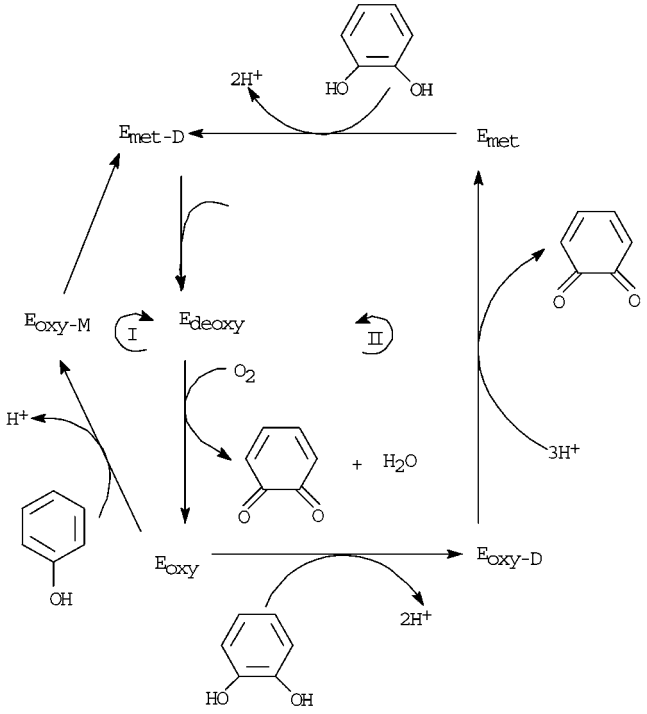


Figure 2. Schematic representation of binuclear copper site. C = Cu ion, O = oxygen, and H = His-N (*6*).

determine the accessibility of various ligands toward the binuclear copper active site, a number of kinetic studies with several compounds (CN^-, phenols, azide, or mimosine) were carried out, and it was found that the large-sized ligands have a higher affinity for the active site as compared to smaller ones (*30*, *31*). Furthermore, this is also supported by the presence of diverse and large-sized substrates/inhibitors of the enzyme tyrosinase (*6*).

2.2. Reaction Mechanism. Tyrosinase catalyzes two distinct oxidation reactions as shown in **Scheme 2**. In cycle I, tyrosinase accomplishes the oxidation of monophenols by oxygen as it passes through four enzyme states (E_{deoxy}, E_{oxy}, E_{oxy-M}, and E_{met-D}); in cycle II, *o*-diphenols are oxidized as the enzyme passes through five enzyme states (E_{deoxy}, E_{oxy}, E_{oxy-D}, E_{met}, and E_{met-D}). The two cycles lead to the formation of *o*-quinones, which spontaneously react with each other to form oligomers (*23*, *32*). A characteristic feature of tyrosinase is a typical lag time related to its monophenolase activity. The hydroxylation of monohydroxyphenols by tyrosinase is as follows: 2 monohydroxyphenols + O_2 + AH_2 → 2 *o*-dihydroxyphenols + H_2O + A, where AH_2 = reductant. Tyrosinase has two separate binding sites in its active center, one for the substrate (monohydroxyphenol) and another for the reductant (*o*-dihydroxyphenol or exogenously added AH_2) (*33*). When exogenous AH_2 is not added, the hydroxylation reaction is characterized by a lag period, which is a dynamic equilibrium between the enzymatic and chemical steps to obtain the steady state with respect to the diphenol concentration (*23*, *34*); to reach such a concentration, a small amount of enzyme must be present in the oxy form (*35*). The lag period is an autocatalytic mechanism, which depends on the elaboration of dopa when tyrosinase acts on tyrosine as the substrate (*36*). Exogenous addition of the

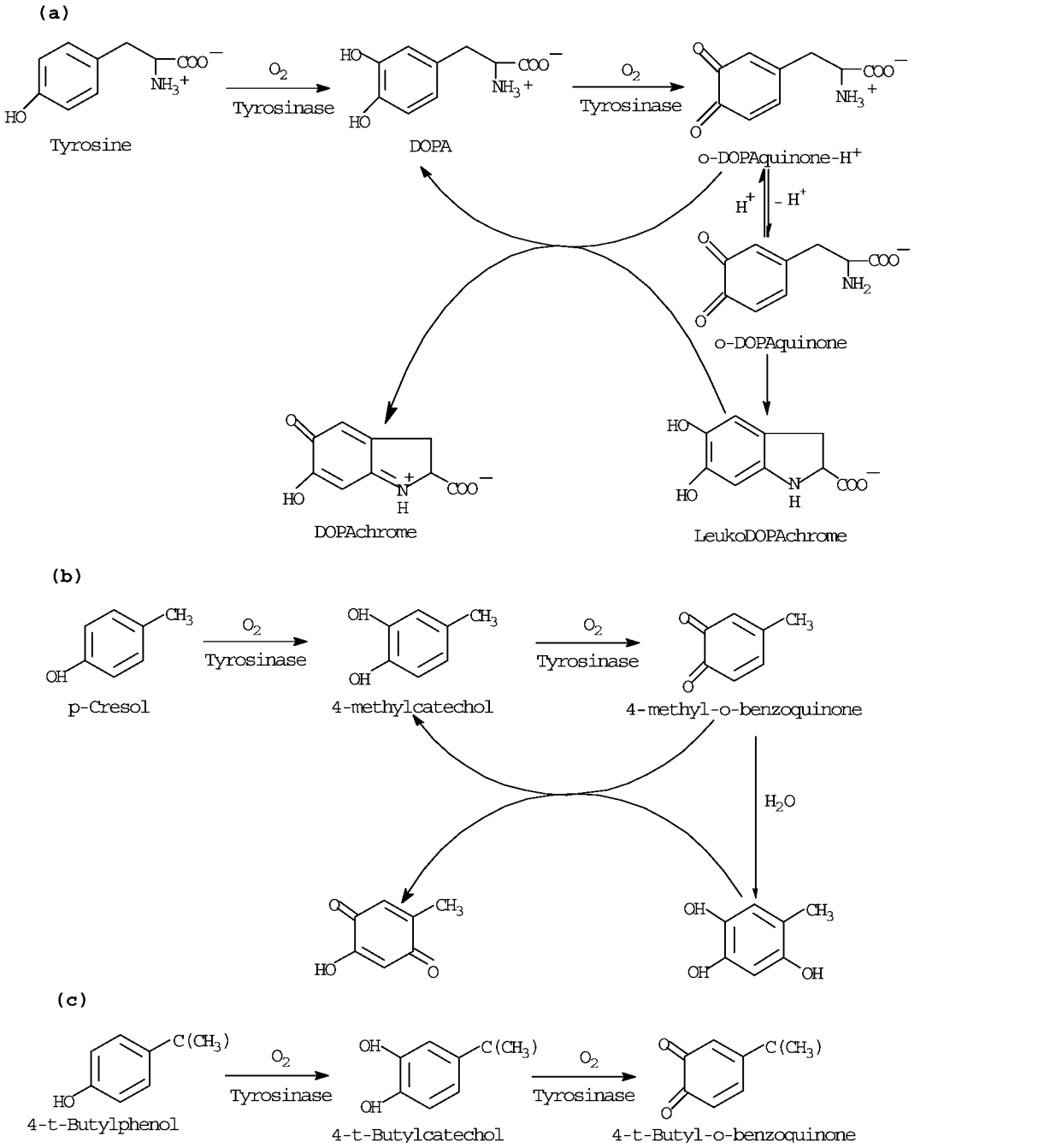
Scheme 2. Catalytic Cycles for the (I) Hydroxylation of Monophenols (M) and (II) Dehydrogenation of *o*-Diphenols (D) to *o*-Quinones by Tyrosinase (*193*)



reductants (ascorbate, hydroxylamine, and hydroquinone) can also shorten the lag period but less effectively than *o*-dihydroxyphenols (*37*). Furthermore, the lag is dependent on various factors such as substrate and enzyme concentration, enzyme source, pH of the medium, presence of a hydrogen donor such as L-dopa or other catechols and transition metal ions (*34*). The absence of a lag period for diphenolase activity can be elaborated by the binding and transformation of *o*-diphenols into *o*-quinones by the E_{met} and E_{oxy} forms, which are present in the activity of the resting tyrosinase (*23*).

2.3. Substrate Stereospecificity. The principal endogenous substrates of mushroom tyrosinase are L-tyrosine, *p*-aminophenol, and its condensation product with glutamate, γ-glutaminyl-4-hydroxybenzene (GHB), all originating from the shikimate pathway (*38*). According to Jimenez and Garcia-Carmona (*39*)

Scheme 3. Reaction Schemes for the Oxidation by Tyrosinase of Three Different Types of Substrates (*39*)



it is possible to divide substrates of tyrosinase into three groups, depending upon the development of *o*-quinonic product (**Scheme 3**):

(i) *o*-Quinone products are cyclizable and sustain intramolecular 1,4-addition to the benzene ring.

(ii) *o*-Quinone products are uncyclizable but can undergo a water addition (*40*).

(iii) *o*-Quinone products are highly stable through the reaction (*35*). Tyrosinase is able to use mono-, di-, and trihydroxyphenols as substrates but has greater affinity for dihydroxyphenols. Furthermore, it was also reported that among the monohydroxyphenols (*p*-cresol and tyrosine), dihydroxyphenols (catechol, L-dopa, D-dopa, catechin, and chlorogenic acid), and trihydroxyphenols (pyrogallol), catechol showed maximum activity, indicating that the enzyme is most active with catechol as substrate (*41*). The stereospecificity of monophenolase and diphenolase activity of mushroom tyrosinase with several enantiomorphs (D-, L-, and DL-tyrosine, methyltyrosine, dopa, methyldopa, and isoprenaline) of monophenols and *o*-diphenols was assayed by Espin et al. (*42*). The lower K_m value observed for L-isomers than for D-isomers indicated stereospecificity in the affinity of tyrosinase toward its substrates. They further elucidated that the phenolic compounds containing electron-wthdrawing groups are poor substrates for tyrosinase as compared to the electron-donating groups. Moreover, affinity properties ($1/K_m$) and catalytic power (V_{max}/K_m) of tyrosinase increase with a decrease in the size of the side chain in the aromatic ring of its substrates.

3. STUDIES ON MUSHROOM TYROSINASE INHIBITORS

Although ~98−99% of the tyrosinase in mushroom is present in its latent form, the remaining active form has a potential for

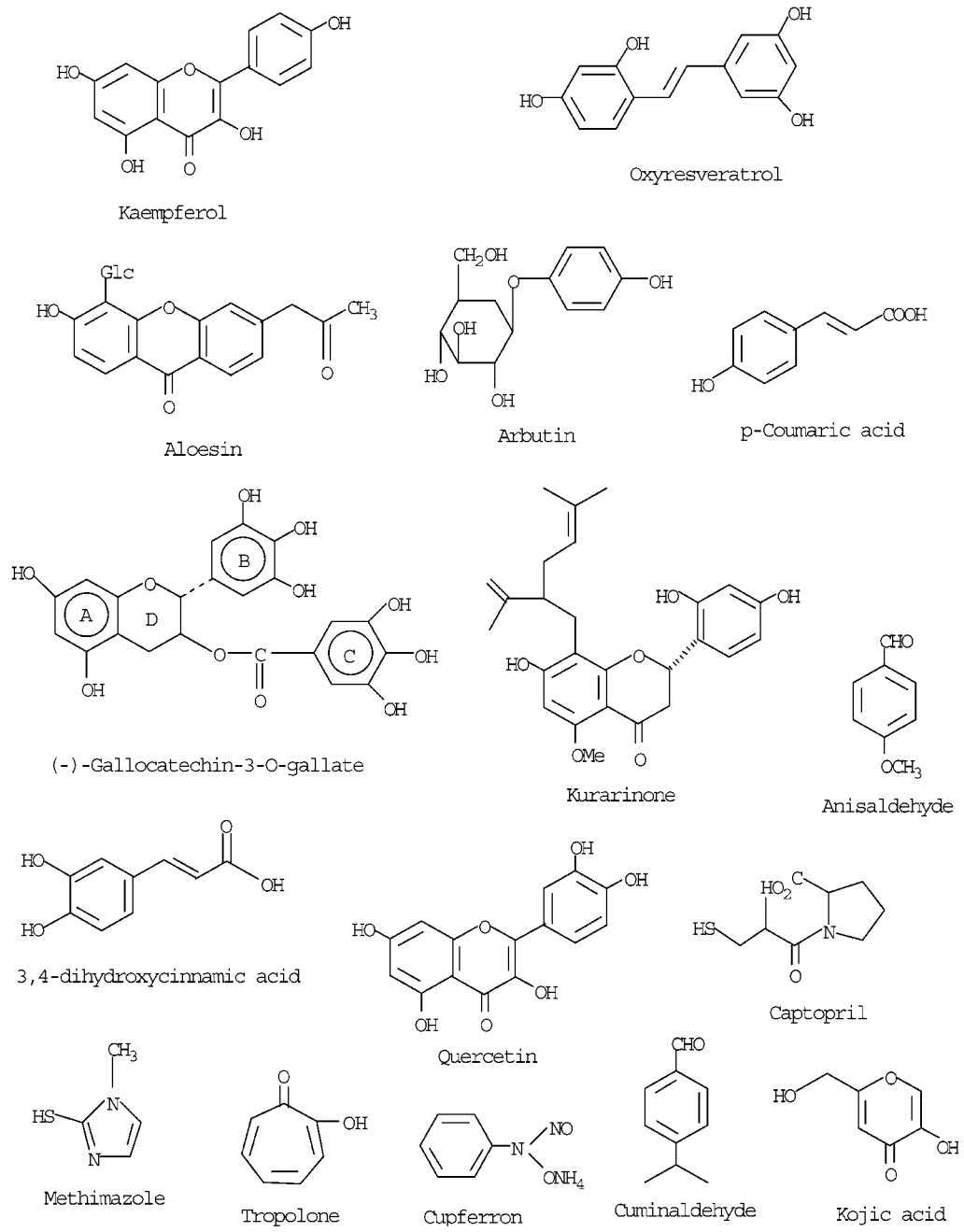


Figure 3. Structures of some mushroom tyrosinase inhibitors.

enzymatic browning during maturity or damage at the time of postharvest handling, which causes severe economic losses to mushroom industries. The most commonly applied inhibitor of the discoloration process currently is sulfite, which, however, is meeting increasing resistance (*43*). Furthermore, tyrosinase inhibitors may be clinically used for the treatment of some skin disorders associated with melanin hyperpigmentation and are also important in cosmetics for skin whitening effects (*44*−*46*), so there is a need to identify the compounds that inhibit mushroom tyrosinase activity. A number of tyrosinase inhibitors from both natural and synthetic sources **(Figure 3**) that inhibited monophenolase, diphenolase, or both of these activities (**Tables 1** and **2**) have been identified.

3.1. Inhibitors from Natural Sources. As plants are a rich source of bioactive chemicals, which are mostly free from harmful side effects, there is an ongoing effort to search for tyrosinase inhibitors from them. A broad spectrum of compounds have been obtained from the natural products and investigated for mushroom tyrosinase inhibitory activity; these compounds differ from one another in the potency and type of inhibition imposed on the enzyme as represented in **Table 1**.

3.1.1. Inhibitors from Higher Plants. A review of the literature indicates that inhibitors are categorized into two main subgroups, namely, polyphenols and aldehydes and other derivatives.

(i) Polyphenols. Polyphenols are a group of chemical compounds that are widely distributed in nature and also known as vegetable tannins because they are responsible for the colors of many flowers. Some of them are complex compounds present in the bark, root, and leaves of plants, whereas others are simple compounds present in most fresh fruits, vegetables, and tea. Some potent tyrosinase inhibitory flavanoides such as kaempferol (*47*−*49*), quercetin (*50*, *51*), kurarinone, and kushnol F (*52*) have been isolated from various plants. A lot of work has been done by Kubo et al. (*53*) to identify and characterize inhibitors from natural sources and to establish the relationship between

Table 1. Some Mushroom Tyrosinase Inhibitors from Natural Sources

inhibitor	source	type of inhibition	ID_{50} (mM)	ref[a]
kaempferol	*Crocus sativus*	competitive[b]	0.230	*49*
quercetin	*Heterotheca inuloides*	competitive[b]	0.070	*49*
kurarinone	*Sophora flavescens*	noncompetitive[c]	0.005	*52*
ECG	green tea	competitive[c]	0.035	*55*
GCG	green tea	competitive[c]	0.017	*55*
EGCG	green tea	competitive[c]	0.034	*55*
1,2,3,4,6-penta-*O*-galloyl-β-D-glucose	*Galla rhois*	noncompetitive[b]	50	*56*
oxyresveratrol	*Morus alba*	noncompetitive[b]	0.001	*62*
anacardic acid	*Anacardium occidentale*	competitive[b]		*60*
p-coumaric acid	*Panax ginseng*	mixed[b]	3.65	*61*
arbutin	*Gvae grsi*	[1]competitive uncompetitive[b]	0.04	*112*
aloesin	*Aloe vera*	[1]noncompetitive	0.10	*112*
3,4-dihydroxycinnamic acid	*Pulsatilla cernua*	noncompetitive[b]	0.97	*68*
4-hydroxy-3-methoxycinnamic acid	,*Pulsatilla cernua*	noncompetitive[b]	0.33	*68*
cuminaldehyde	cumin seed	noncompetitive[b]	0.05	*67*
cumic acid	cumin seed	noncompetitive[b]	0.26	*67*
anisaldehyde	anise oil	noncompetitive[b]	0.38	*68*
anisic acid	anise oil	noncompetitive[b]	0.68	*68*
trans-cinnamaldehyde	*Cinnamomum cassia*	competitive[b]	0.85	*63*
(2*E*)-alkenal (C_7)	*Oliva olea*	noncompetitive[b]	1.3	*64*
2-hydroxy-4-methoxybenzaldehyde	*Mondia whitei*, *Rhus vulgaris*, *Scleroca caffra*	mixed[b]	0.03	*65*
Ia	*Agaricus hortensis*	competitive[b]		*74*
Ib	*Agaricus hortensis*	noncompetitive[b]		*74*
agaritine	*Agaricus bisporus*	uncompetitive[b] competitive[c]		*76*
metallothionein	*Aspergillus niger*	mixed[d] mixed[e]	0.22 20.2	*75*

[a] Reference cited for ID_{50} values. [b] wrt dopa. [c] wrt tyrosine. [d] wrt catechin. [e] wrt chlorogenic acid.

Table 2. Some Mushroom Tyrosinase Inhibitors from Synthetic Sources

inhibitor	type of inhibition	ID_{50} (mM)	ref[a]
cinnamaldehyde	noncompetitive[b]	0.97	*68*
cinnamic acid	mixed[b]	0.70	*68*
captopril	noncompetitive[c] competitive[b]		*77*
methimazole	mixed[b]		*78*
cupferron	competitive[b]	0.001	*81*
tiron		400	*83*
2-methoxycinnamic acid	noncompetitive[b]	0.34	*68*
3-methoxycinnamic acid	noncompetitive[b]	0.35	*68*
4-methoxycinnamic acid	noncompetitive[b]	0.34	*68*
4-substituted benzaldehydes	competitive[b]		*72*
L-mimosine	competitive[c]		*86*
kojic acid	mixed[b]	0.014	*52*
tropolone	competitive[b]		*88*
4-substituted resorcinol	competitive[b]		*73*
dimethyl sulfide	competitive[b]		*92*
benzoic acid	mixed[b]	0.64	*67*
benzaldehyde	noncompetitive[b]	0.82	*67*
p-hydroxybenzaldehyde	competitive[b]	1.2	*64*
citral	noncompetitive[b]	1.5	*64*

[a] Reference cited for ID_{50} values. [b] wrt dopa. [c] wrt tyrosine.

their inhibitory activity and structure. According to them, all flavanoides inhibit the enzyme due to their ability to chelate copper in the active site. However, this condition is applicable only if the 3-hydroxy group is free. They further elucidated that the 3-hydroxy group is not an essential requirement for inhibition as other types of flavonoids such as luteolin 4′-*O*-glucoside and luteolin 7-*O*-glucoside, lacking this 3-hydroxy group, still showed inhibitory activity (*48*). Recently, Badria and el Gayyar (*54*) found that flavonoids containing an α-keto group possess potent tyrosinase inhibitory activity. This may be explained in terms of similarity between the dihydroxyphenyl group in L-dopa and the α-keto group in flavonoids. The results of this study revealed a new type of tyrosinase inhibitor from natural origin. Application of these compounds will further be examined for treatment of hyperpigmentation. Another important compound of this group is gallic acid, which occurs as multiple esters with D-glucose, and their esters are widely used as additives in food industries. Various gallic acid derivatives have been isolated from green tea (*55*) and *Galla rhois* (*56*), and some of them were identified as strong tyrosinase inhibitors. Studies indicate that the flavon-3-ol skeleton with a galloyl moiety at the 3-position is an important structural requirement for optimum inhibition of tyrosinase activity. It is interesting to note that 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (PGG), the active compound isolated from *G. rhois* (*56*), has a potent tyrosinase inhibitory activity, although this is not consistent with previous reports that the tyrosinase inhibitory strength of aromatic carboxylic acids decreases with the esterification, hydroxylation, or methylation of the benzene ring (*57*, *58*). However, gallic acid and its short alkyl (<C10) chain esters were oxidized by tyrosinase as substrates, yielding the yellow oxidation products, but the long alkyl (>C10) chain esters inhibited the enzyme without producing the pigmented products, indicating that the carbon chain length is related to their tyrosinase inhibitory activity. In other words, the gallates with increasing hydrophobicity of the molecules become more resistant to being oxidized by the enzyme due to disruption of the tertiary structure of the enzyme (*59*). In various other bioactive constituents such as cardol derivatives (*60*), addition of a hydroxy group increased whereas addition of a methyl group decreased the inhibitory activity, and also the unsaturated alkyl side chain exhibited stronger inhibition as compared to the saturated one. It was observed that *p*-coumaric acid (*61*) inhibited both monphenolase and diphenolase activities and a polar hydroxy group at the para position increased the monophenolase inhibitory activity, whereas it decreases diphenolase inhibitory activity. Strong tyrosinase inhibitory activity was reported by oxyresveratrol (*62*), due to the presence of a maximum number of hydroxy groups in the ring. However, a

clear explanation regarding their inhibitory activity and structure criteria is lacking.

(ii) Aldehyde and Other Derivatives. A large number of aldehydes and other derivatives were also isolated and characterized as mushroom tyrosinase inhibitors such as *trans*-cinnamaldehyde (*63*), (2*E*)-alkenals (*64*), 2-hydroxy-4-methoxybenzaldehyde (*65*), anisaldehyde (*66*), cuminaldehyde and cumic acid (*67*), and 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid (*68*). As the aldehyde group is known to react with biologically important nucleophilic groups such as sulfhydryl, amino, and hydroxy groups, so it has been proposed that its inhibitory effect is due to the formation of a Schiff base with the primary amino group of the enzyme. Comparison of the inhibitory activities of various aldehydes and closely related compounds such as cinnamic acid, anisic acid, cumic acid, and benzoic acid proved cuminaldehyde to be the strongest inhibitor (*67*). It is interesting to note that electron-donating groups (isopropyl and methoxy) at the para position in cuminaldehyde provide stability to the Schiff base at the active site of the enzyme through inductive effect. In the case of (2*E*)-alkenals the hydrophobic alkyl chain length from the hydrophilic enal group seems to be related to their inhibitory potency, which may be due to better association of the longer alkyl chain with the hydrophobic protein pocket close to the binuclear copper site (*69*−*71*). Except for 2-hydroxy-4-methoxybenzaldehyde, the above-indicated aromatic aldehydes were described as noncompetitive tyrosinase inhibitors by Kubo and Kinst-Hori (*64*, *66*, *67*). However, a contradictory statement regarding mode of inhibition of the aldehydes was recently reported by Jimenez et al. (*72*), according to which all of the 4-substituted benzaldehyde derivatives behave as competitive inhibitors of L-dopa oxidation. In the case of acid derivatives, the mechanism of inhibition involves the formation of a copper−carboxylic acid complex at the binuclear copper site of the enzyme (*71*); further substitution of a phenolic group at the para position increased the extent of inhibition (*70*).

Most of the above inhibitory studies were made on the basis of ID_{50} values, a constant to determine extent of inhibition, which indicates the inhibitor concentration required for 50% inhibition. However, it is not a valid parameter for some kinds of tyrosinase inhibitors (*73*) and also is related to only diphenolase activity. Therefore, more reliable kinetic parameters are required to evaluate both the mono- and diphenolase activities of tyrosinase.

3.1.2. Inhibitors from Fungi. Besides higher plants, some compounds from fungal sources have also been identified and reported for their inhibitory activity toward mushroom tyrosinase. Madhosingh and Sundberg (*74*) isolated, purified, and characterized two inhibitors from mushroom *Agaricus hortensis*. Inhibitor **Ia** inhibited the enzyme competitively, whereas **Ib** noncompetitively inhibited the enzyme, as revealed by the Lineweaver−Burk plots. Metallothionein from *Aspergillus niger* (*75*) has strong avidity to chelate copper at the active site of mushroom tyrosinase, thereby acting as a strong inhibitor. Another possible mechanism for such an inhibitory effect could be explained by the presence of sulfhydryl amino acids in the metallothionein, which bind with *o*-quinones to form colorless thioesters. Recent reports showed that agaritine from *A. bisporus* inhibited mushroom tyrosinase in vitro and displayed uncompetitive inhibition of L-dopa and competitive inhibition of L-tyrosine, implying that agaritine is metabolized by the monophenolase activity (*76*).

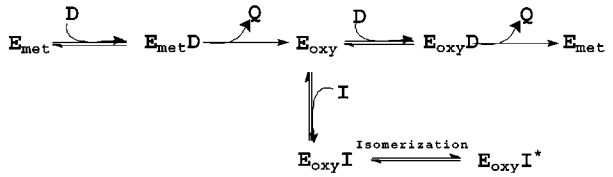
3.2. Inhibitors from Synthetic Origin. A variety of inhibitors reported from synthetic origin are listed in **Table 2**. Interestingly, some of these inhibitors are from drugs, whereas other are simple chemicals.

(i) Drugs. Captopril, an antihypertensive drug [(2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline], is able to prevent melanin formation (*77*) by irreversibly inhibiting both mono- and diphenolase activities of mushroom tyrosinase in noncompetitive and competitive manners, respectively, as well as by scavenging the generated *o*-quinones to form a colorless conjugate. The inhibition of both monophenolase and diphenolase activities of tyrosinase by captopril showed positive kinetic co-operativity, which arose from the protection of both substrate and *o*-quinone against inhibition by captopril. The drug forms both a copper−captopril complex and a disulfide bond between captopril and cysteine-rich domains at the active site of the enzyme. An antithyroid drug, methimazole (1-methyl-2-mercaptoimidazole), that acts as a tyrosinase inhibitor affects both mono- and dihydroxyphenolase activities of mushroom tyrosinase (*78*). It is interesting to note that both drugs interacted with mushroom tyrosinase in a similar manner but the types of inhibition were different.

(ii) Chemicals. A number of chemicals such as hydrogen peroxide, hydroxylamine, Tiron, thiols, and aromatic carboxylic acids have been reported for their anti-tyrosinase activity. Besides these, some other chemicals are also reported and classified as slow binding inhibitors depending upon their action mechanism. Hydrogen peroxide (H_2O_2) inactivates mushroom tyrosinase in a biphasic manner, with the rate being faster in the first phase than in the second (*79*). Enzyme inactivation is dependent on H_2O_2 concentration and independent of pH, and inhibition is faster under anaerobic condition than under aerobic one. Copper chelators (tropolone and sodium azide) and substrate analogues (L-mimosine, L-phenylalanine, *p*-fluorophenylalanine, and sodium benzoate) protect the enzyme against inactivation by H_2O_2, indicating that Cu^{2+} at the active site of mushroom tyrosinase is essential for inactivation by H_2O_2. Another inhibitor, hydroxylamine, at low concentration (33 mM) shortens the lag period of tyrosine hydroxylation, whereas the relatively high concentration (>20 mM) inhibits *o*-dihydroxyphenolase activity and lowers the extent of final pigment production, the inactivation rate being faster under anaerobic condition. It was found that NH_2OH changes the spectra of *o*-quinones, which is attributed to oxime formation. As a result, apparent inhibition exerted by NH_2OH on the *o*-dihydroxyphenolase activiy is due to both spectral changes in pigmented product formation and inactivation of the enzyme by NH_2OH (*80*). The most powerful tyrosinase inhibitor among N-substituted *N*-nitosohydroxylamines was found to be *N*-cyclopentyl-*N*-nitrosohydroxylamine ($IC_{50} = 0.6\ \mu M$). The removal of a nitroso or hydroxy moiety resulted in total loss of enzyme inhibitory activity, suggesting that both of these groups are essential for activity, probably by interacting with the copper ion at the active site of the enzyme (*81*). The compound Tiron has shown multiple effects on mushroom tyrosinase at various concentrations. At low concentration, Tiron acts as a weak reductant as compared to L-dopa, so it prevents the few L-dopa molecules endogenously formed during tyrosine hydroxylation from accessing the enzyme, which are required as reductant for the hydroxylation reaction (*82*), thereby extending the lag period of tyrosine hydroxylation. However, at higher concentrations it acts as an effective reductant, thus shortening the lag period of tyrosine hydroxylation (*83*).

Cysteine and a wide range of aromatic carboxylic acids were inhibitors for the activity of commercially purified mushroom tyrosinase. Competitive, noncompetitive, mixed, or uncompeti-

Scheme 4. Action Mechanism for Slow Binding Inhibitors (*92*)[a]



[a] D = *o*-diphenol, Q = *o*-quinone, and I = inhibitor. Inhibitor binds to the oxy form of the enzyme and slowly isomerizes to give an enzyme–inhibitor complex ($E_{oxy}I^*$).

tive inhibitions were obtained depending on the nature of the inhibitor and the method used for the determination of the enzyme activity (*58*). Lindbladh et al. (*84*) studied the effect of the catalase on the inactivation of tyrosinase by ascorbic acid, cysteine, and glutathione. Ascorbic acid inactivated tyrosinase, but addition of catalase prevented this inactivation and it was found to be dependent on oxygen, whereas inactivation by cysteine and glutathione was independent of the oxygen and addition of catalase increased enzyme inactivation at high thiol concentrations and the inactivation was independent of oxygen. Large amounts of serum albumin protected tyrosinase from inactivation by ascorbic acid but did not prevent inactivation by cysteine/glutathione. Benzoic acid inhibited α, *β*, and *γ* isozymes of *A. bisporus* tyrosinase competitively for cresolase reaction, but showed partial uncompetitive inhibition for α and *β* isozymes and a simple competitive inhibition for *γ* isozyme in catecholase reaction (*57*). Dihydroxybenzoic acids (DBA) such as 3,4-DBA, 3,5-DBA, and 2,4-DBA inhibited L-dopa oxidation by mushroom tyrosinase (*85*). 2,3-DBA and 2,5-DBA at relatively low concentrations had a synergistic effect on the reaction due to the ability of their *o*-quinones to oxidize DL-dopa nonenzymetically, whereas at higher concentration they inhibited the rate of DL-dopa oxidation.

Another group of compounds such as L-mimosine (*86*), kojic acid (*87*), tropolone (*88*), and 4-substituted resorcinols (*73*), having structural similarity to phenolic substrates and showing competitive inhibition with respect to these substrates, are known as slow binding inhibitors (*89*−*91*). The reaction mechanism of these inhibitors is represented in **Scheme 4** according to which all of these slow binding inhibitors compete with a second molecule of L-dopa to bind to the oxy form of the enzyme, which is an obligatory intermediate in the catalytic turnover, and thus the presence of the substrate is necessary for the action mechanism of slow binding inhibitors. Recently, dimethyl sulfide (DMS) was also reported as a slow binding competitive inhibitor of mushroom tyrosinase and is the first volatile inhibitor of tyrosinase to be characterized (*92*). DMS has a physiological role within plant tissues as its high concentration inhibits endogenous tyrosinase, thereby protecting the plant from premature phenolic oxidation.

3.3. Importance of Mushroom Tyrosinase Inhibitors. 3.3.1. Food Industry. Browning in fruits and vegetables is of great concern to growers and the food industry as it impairs the organoleptic properties of the product. The rate of enzymatic browning depends on the concentration of active tyrosinase and phenolic compounds, oxygen availability, pH, and temperature conditions in the tissue (*22*). Thus, it is necessary to identify various methods to stop enzymatic browning caused by tyrosinase. The current conventional techniques to avoid browning include use of autoclave and blanching methods to inactivate tyrosinase, but these processes cause important weight and nutrient losses in the product (*93*). Another alternative approach is the use of microwave energy, but it also suffers from the main disadvantage that a temperature gradient is generated within the sample (*94*), which causes enzyme inactivation in overheated regions only and in colder regions the enzyme may not be completely inactivated; moreover, it causes internal water vaporization with associated damage to the mushroom texture (*95*). The application of a combined microwave−hot water treatment is slightly better in terms of final product quality (*96*). In any case, microwave blanching is not very successful in the food industry except for some isolated applications. Various chemicals such as halide salts, aromatic carboxylic acids (*97*, *98*) and other compounds with reducing properties such as sulfite (*99*), ascorbic acid and its derivatives (*100*, *101*), and thiol compounds such as cysteine (*102*, *103*) are known to inhibit tyrosinase. The effect of ascorbic acid, sodium bisulfate, and other reducing agents on tyrosinase has been controversial over the years (*104*); moreover, the use of sulfites is becoming more and more restricted due to potential health hazards (*43*). Other alternative methods were also proposed such as formulation without sulfite (*105*) or new products with a stabilized form of ascorbic acid and *β*-cyclodextrins (*106*) and plant sulfhydryl proteases (*107*). Presently, the use of 4-hexylresorcinol is considered to be safe in the food industry and is quite effective in prevention of shrimp melanosis (*108*, *109*) and for browning control in fresh and dried fruit slices (*110*). However, as safety is of prime concern for an inhibitor to be used in food industry, there is a constant search for better inhibitors from natural sources as they are largely free of any harmful side effects. Many of the inhibitors described in this review are flavor condiments and are listed as food flavor ingredients in *Fenaroli's Handbook of Flavor Ingredients* (*111*), which makes their use favorable in food industries.

3.3.2. Cosmetic Industry. Use of tyrosinase inhibitors is becoming increasingly important in the cosmetic industry due to their skin-whitening effects. A number of tyrosinase inhibitors are reported from both natural and synthetic sources, but only a few of them are used as skin-whitening agents, primarily due to various safety concerns. For example, linoleic acid, hinokitiol, kojic acid, naturally occurring hydroquinones, and catechols were reported to inhibit enzyme activity but also exhibited side effects (*46*). Currently, arbutin and aloesin are used in the cosmetic industry as whitening agents because they show strong inhibition toward the tyrosinase enzyme, which is responsible for pigmentation in human beings. Arbutin, a hydroquinone glycoside, and aloesin, a C-glycosylated chromone, were isolated from leaves of *Gvae grsi* and *Aloe vera*, respectively, and studied for their inhibitory effects (*112*). Arbutin was reported to inhibit the enzyme activity competitively (*113*), whereas in another contrasting report Funayama et al. (*114*) suggested that of two forms of arbutin only *β*-arbutin inhibited both tyrosinase activities from mushroom and mouse melanoma noncompetitively and that α-arbutin inhibited only the tyrosinase from mouse melanoma by mixed-type inhibition. In addition, Jin et al. (*115*) studied the effect of cotreatment of aloesin and arbutin and found that both inhibit tyrosinase activity in a synergistic manner by acting through different mechanisms; aloesin inhibits noncompetitively, whereas arbutin inhibits competitively. Taken together they inhibit melanin production synergistically by a combined mechanism of noncompetitive and competitive inhibitions. Thus, all of the above findings indicate that it is beneficial to use aloesin and arbutin as a mixture for depigmentation effect because the cotreatment cuts down the effective doses of these agents for the same inhibitory effect on tyrosinase activity and can reduce adverse side effects.

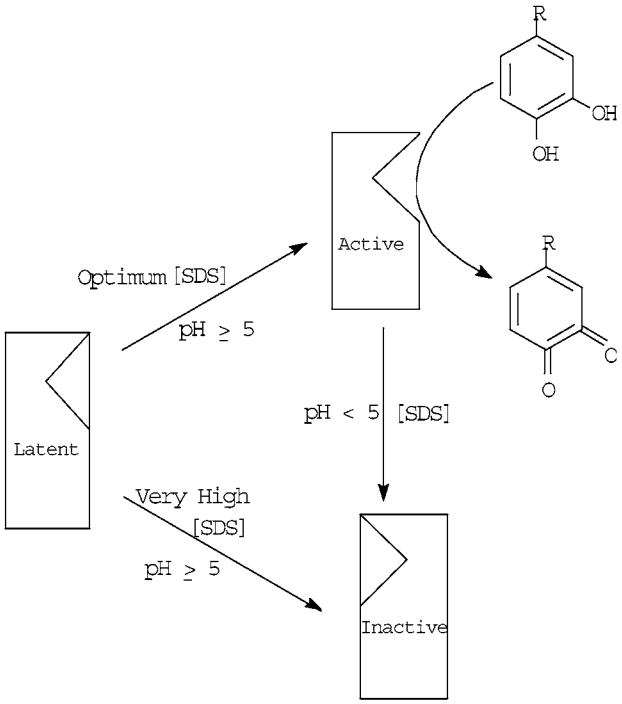


Figure 4. pH-dependent activation of latent mushroom tyrosinase by SDS (*130*).

4. STUDIES ON MUSHROOM TYROSINASE ACTIVATORS

The enzyme tyrosinase can be found in either latent or active form as reported above; the latent tyrosinase represents ~99% of total tyrosinase activity in mushrooms (*116*). Tyrosinases can be activated by a broad spectrum of substances, in crude tissue preparation. The activation of latent tyrosinase from plant and insect sources has been reported by different treatments or agents such as anionic detergents such as SDS (*117*−*120*), acid shock (*121*, *122*), fatty acids (*123*, *124*), alcohols (*125*), proteases (*126*−*128*), and pathogen attack (*129*). However, there are very few reports on the activation of latent tyrosinase from mushroom as source material. Recently, activation of mushroom tyrosinase by SDS (*130*), benzyl alcohol (*131*), and serine protease (*132*) has been reported. The activation process is characterized by the presence of a lag period (τ) prior to the attainment of steady-state rate, suggesting that the activation could take place through a slow conformational change of the enzyme to render the active tyrosinase. Optimum SDS concentration and pH $\geq$5 results in activation of the enzyme, whereas very high SDS concentration and pH $\geq$5 causes further protein denaturation; in the presence of SDS and pH $<$5 the active form of the enzyme is converted to an inactive one **(Figure 4**). The same reaction mechanism operated in protease-treated tyrosinase isoforms, despite their different kinetic features (*132*). Latent mushroom tyrosinase appears to be sensitive to activation preparations containing tolaasin (*129*), which is a bacterial lipodepsipeptide produced by the casual agent of brown blotch disease, *Pseudomonas tolaasii* (*133*). Another activator, 3-hydroxyanthranilic acid (HAA), was found to affect the enzyme activity by mimicking the effect of true diphenol, thereby acting as a diphenol substrate (*134*). HAA significantly affects the monophenolase activity of tyrosinase by shortening its lag time and increasing the catalytic activity of the enzyme. The activation brought about by several seemingly unrelated substances is a phenomenon often attributed to conformational changes of the enzyme molecule, solublization of the enzyme, or removal of an inhibitor.

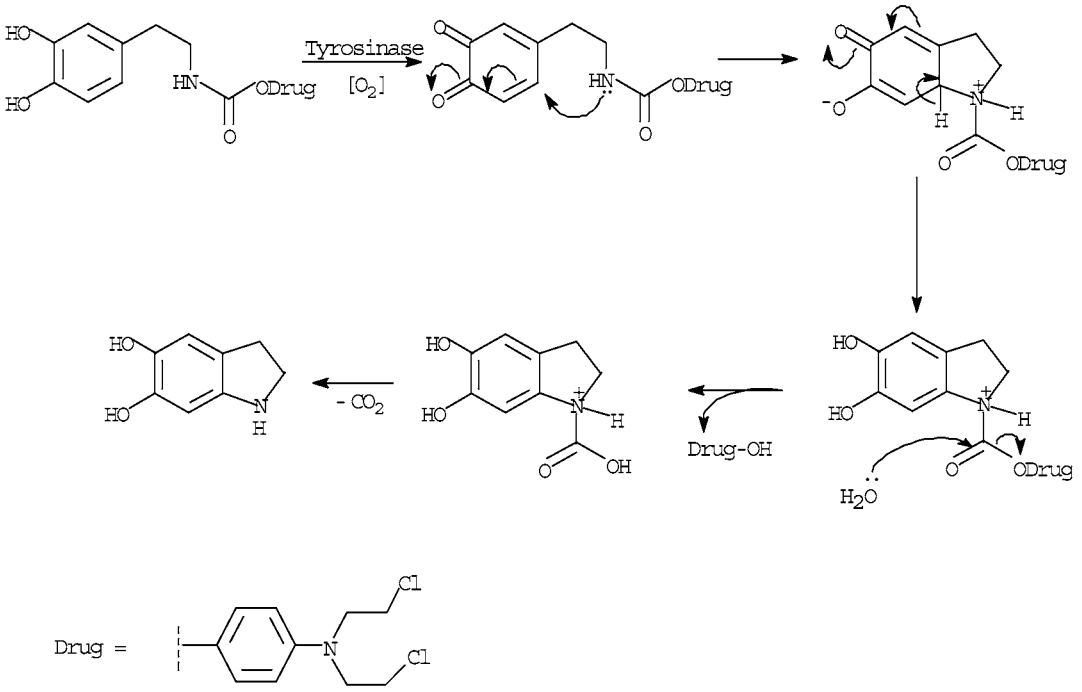
5. CLINICAL STUDIES ON MUSHROOM TYROSINASE

For many years mushroom tyrosinase has been studied for its use in cosmetics as well as in food industries. However, various recent papers have exposed some previously unexplored aspects of mushroom tyrosinase in clinical studies. Some of them are described below.

5.1. Marker of Vitiligo. Vitiligo is an autoimmune disease, characterized by hair hypopigmentation and total melanocyte depletion in the basal layer of the epidermis. Tyrosinase is the enzyme responsible for melanin production in normal melanocytes and melanoma cells and is known to be an autoantigen in various autoimmune disorders. Immunological aspects of vitiligo showed the generation and presence of autoantibodies directed against melanocyte antigens in the patients' sera. Using solid-phase ELISA on mushroom tyrosinase, higher titers of IgG anti-tyrosinase antibodies were found in patients with diffused vitiligo as compared to localized vitiligo. These anti-tyrosinase autoantibodies from vitiligo patients' sera can be recovered by exploiting its affinity toward tyrosinase. These antibodies neither cross-react with other autoantigens in different autoimmune disorders nor block tyrosinase activity, which shows that they are not reacting with the catalytic site of the enzyme. This indicates that tyrosinase acts as an autoantigen and serves as a marker for vitiligo (*135*). In an attempt to prevent melanocyte destruction Zehtab et al. (*136*) administered tyrosinase from *A. bisporus* orally in animal models, which resulted in diminished cell-mediated immune response, and it was suggested that this oral administration is closely linked to suppression of cellular response to autoantigens (*137*, *138*). Therefore, it will be useful in longitudinal studies to determine the relationship between the clinical features of vitiligo and tyrosinase antibody levels.

5.2. Role in Cancer. Contradictory results are available regarding the role of tyrosinase in cancer as some papers suggest a tumor-suppressing effect of mushroom tyrosinase, whereas others predict a possible role in mutagenicity. Vogel et al. (*139*) reported that a stable phenol, γ-L-glutaminyl-4-hydroxybenzene (GHB), is oxidized by tyrosinase to a quinone and a second oxidation product, which together suppress mitochondrial energy production and synthesis of nucleic acids and proteins. Incubation of cultured murine L1210 leukemia and B-16 melanoma cells with purified quinone blocked tumor growth in the mice, but when these cells were incubated in the presence of GHB, tumor suppression was observed only in B-16 melanoma cells and not in L1210 leukemia cells due to the absence of the enzyme tyrosinase, indicating that the cytotoxic effect of GHB is dependent on the presence of tyrosinase. The antitumor effect of L-glutamic acid and γ-(*p*-hydroxyanilide), on B-16 melanoma was studied in vivo. In the presence of mushroom tyrosinase it inhibited DNA polymerase activity and its 3,4-dihydroxy derivative inhibited thymine, whereas the 2,5-dihydroxy derivative inhibited uracil and leucin incorporation into nucleic acid and proteins of melanoma cells (*140*). However, other results indicate a negative aspect of the mushroom tyorsinase toward cancer. Papaparaskeva-Petrides et al. (*141*) found that tyrosinase is responsible for enhancing the mutagenicity of mushroom extract due to production of phenolic and quinoid compounds. Moreover, this mutagenic response was inhibited by catalase, superoxide dismutase, glutathione, and dimethyl sulfoxide, which indicates the role of phenolic and quinoid compounds in the generation of reactive oxygen species. A similar increase in mutagenicity was also observed with baked mushroom extract (*142*). Aromatic hydrazines play an important role in the carcinogenicity of mushroom (*143*, *144*), so a number of studies have been performed to find the relationship between hydrazines

Scheme 5. Tyrosinase-Mediated Drug Release Mechanism (*151*)



and mushroom tyrosinase. The principal hydrazine considered to be a candidate for mediating the carcinogenicity of the mushroom is agaritine [β-*N*-(γ-L-(+)-glutamyl)-4-(hydroxymethyl)phenylhydrazine]. Walton et al. (*145*) studied the mutagenicity of putative agaritine metabolites in the presence of mushroom tyrosinase and found that among the metabolites tyrosinase enhanced the mutagenicity of *N*′-acetyl-4-(hydroxymethyl)phenylhydrazine. Recently, the same co-workers reported that the whole mushroom homogenate readily metabolizes agaritine, whereas the mushroom tyrosinase has the potential to metabolize both agaritine and *N*′-acetyl-4-(hydroxymethyl)-phenylhydrazine, in the latter case forming genotoxic metabolites (*146*). Agaritine is bioactivated by the loss of the γ-glutamyl group, catalyzed by γ-glutamyl transpeptidase, to release free hydrazine [4-(hydroxymethyl)phenylhydrazine], which is further oxidized to generate the 4-(hydroxymethyl)benzene diazonium ion. It is interesting to note that the mutagenicity of agaritine is much lower that that of its metabolite, 4-(hydroxymethyl)-benzene diazonium ion (*145*). The contribution of this pathway to the mutagenicity of ethanolic mushroom extracts (*141*, *147*) or the metabolism and/or carcinogenicity of hydrazines in animals remains to be elucidated.

5.3. Prodrug Therapy. Malignant melanoma continues to be a serious clinical problem with a high mortality rate among the human beings and this high mortality rate is due to the failure of melanoma cells to respond to cytotoxic treatment in the form of radiation and chemotherapy. Thus, the metastatic melanoma continues to challenge the researchers to find a systemic treatment of cancer. To develop such a treatment with a selective cytotoxic response, it is necessary that it should interfere with the biosynthetic pathway, which converts tyrosine into melanin (*148*, *149*) by tyrosinase. This would allow selective conversion of inactive prodrugs, modeled on tyrosine, into cytotoxic drugs in melanoma cells. Such a selective strategy toward the treatment of malignant melanoma is called melanocyte-directed enzyme prodrug therapy (MDEPT), which offers a highly selective drug delivery system (*150*, *151*). Various cytotoxic agents such as phenol mustard (*152*), bis(ethylamine) mustard (*153*), and daunorubicin (*154*) were examined for their ability to be oxidized by mushroom tyrosinase as oxidation by enzyme is the first step in the prodrug release mechanism (**Scheme 5**) (*151*). It was observed that the prodrug, which has close structural resemblance with natural tyrosinase substrate, is an appropriate MDEPT candidate. The structural alteration (functional group transformation or removal of steric bulky group via heteroatom) at the substrate's oxidative site decreases the rate of tyrosinase-catalyzed oxidation. In addition to the above clinical applications, Ourth and Renis (*155*) were able to generate antiviral activity against herpes simplex virus-1 using hemolymph phenol oxidase or mushroom tyrosinase under in vitro conditions.

5.4. Antioxidant Properties. The oxidation process has an important role in energy production to fuel biological processes in almost all living tissues. Oxygen free radicals are produced during normal metabolism and at low concentrations have a useful role in moduating gene expression and signal transduction, but a high concentration of these radicals is extremely harmful to DNA and other macromolecules (*156*−*158*). In living beings the uncontrolled production of free radicals leads to many diseases such as cancer, atherosclerosis, and rheumatoid arthritis as well as degenerative processes related with aging (*159*). However, the presence of antioxidants in the diet helps to reduce oxidative damage. Since ancient times mushroom has been used as an essential component of the diet, so a number of studies have been made to investigate its antioxidant potential. Recently, Shi et al. (*160*) have reported that the cold-water extracts of *A. bisporus* prevented H_2O_2-induced oxidative damage to cellular DNA but were unable to identify the nature of the protective mechanism. Later they (*161*) were able to correlate the genoprotective effect of *A. bisporus* with a heat labile protein (FII β-1), identified as tyrosinase, and the nature of the genoprotective activity of tyrosinase was found to be dependent upon the hydroxylation of tyrosine to L-dopa and subsequent oxidation of L-dopa to dopaquinone (*162*). This is quite interesting because L-dopa is normally linked with toxic pro-oxidant properties as it produces highly unstable electrophilic dopa-(semi)quinones. On redox cycling these quinones produce harmful oxy radicals, peroxides, semiquinones, and quinones

(*163*, *164*), which are responsible for antitumor activity and neurotoxic damage in Parkinson's disease (based on treatment with L-dopa). Furthermore, it was also found that the generation of oxygen free radicals, produced from L-dopa in the presence of Cu(II), is related with strand breakage of DNA under in vitro conditions (*165*, *166*). However, the exact mechanism of the genoprotective effect of the L-dopa oxidation product generated by tyrosinase is not completely understood. Besides having prooxidant activity, L-dopa also stimulates a cellular antioxidant defense mechanism under certain conditions. At low concentrations it increased intracellular concentrations of the antioxidant glutathione, thereby enhancing the free radical scavenging capacity of the brain cells (*167*−*169*).

6. STUDIES ON IMMOBILIZATION OF MUSHROOM TYROSINASE

Enzyme immobilization is one of the important aspects of biotechnology, and recent trends shows that it is equally viable in industries, too, as it lowers the production cost due to the reusability of the enzymes. The various techniques used for enzyme immobilization include entrapment in polymeric gels, adsorption onto insoluble materials, encapsulation in membranes, cross-linking with bifunctional or multifunctional reagents, and linking to an insoluble carrier (*170*).

A number of attempts have been made by the various researchers to explore future implications of entrapped tyrosinase for commercial production of L-dopa and for that tyrosinase has been immobilized on cellulose support (*171*), collagen membrane (*172*), polyacrylamide gel (*173*), CH-Sepharose (*174*), Enzacryl AA (*175*), copper−alginate gel (*176*), nylon tubing (*177*), hydroxyaluminum−montmorillonite complex (*178*), and chitosan flakes using glutaraldehyde as cross-linking agent (*179*) under various conditions. Recently, Munjal and Sawhney (*180*) immobilized mushroom tyrosinase by entrapment in alginate, polyacrylamide, and gelatin gels and reported that the enzyme entrapped in gelatin has a higher activity yield, a greater storage life, and better thermal stability and, thus, can be used for large scale L-dopa production, which is a commonly prescribed drug for the treatment of Parkinson's disease.

In addition to the above industrial application, some coworkers used mushroom tyrosinase as a catalyst for the removal of phenolic compounds from wastewater, (*181*) and the extent of transformation is substrate dependent (*182*). The addition of aluminum sulfate had little effect on the removal of colored products from phenol solutions treated with tyrosinase. All treated solutions of phenol and chlorophenols, except 2,4-dichlorophenol, had substantially lower toxicities with tyrosinase as compared to other peroxidase enzymes (*183*). Tyrosinase can be effectively used for bioconversion of phenolic substrates by covalent attachment to polymers (ethylene glycol derivatives), which increase enzyme stability (*184*). In the treatment of phenols using tyrosinase a color change was observed, which can be removed by cotreatment of tyrosinase with amino group containing polymer such as chitosan, hexamethylenediamine−epichlorohidrin, and polyethyleneimine, the latter being more effective than chitosan. Immobilizing tyrosinase on cation exchange resins and magnetite resulted in almost complete removal of phenols (*185*, *186*). A two-step approach was employed by Sun et al. (*187*) in which, first, phenols were converted to quinones by tyrosinase and then chemisorbed onto chitosan, which resulted in effective removal of almost all UV-absorbing materials from the effluent. These results show that immobilized tyrosinase gave better results than free enzyme.

Furthermore, for quantification and better removal of phenols, biosensors have been developed using immobilized tyrosinase,

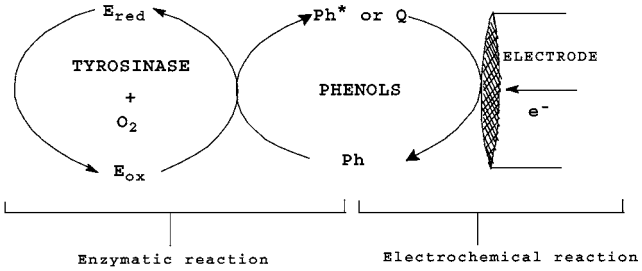


Figure 5. Reaction sequence for tyrosinase biosensor. Ph = phenol, Ph* = phenoxy radical, and Q = quinone (*190*, *191*).

which exploit the enzyme−substrate affinity for the detection of pollutants in environmental samples. Numerous biosensors proposed for the detection of phenolic compounds are based primarily on the phenol oxidizing enzyme, tyrosinase (*188*, *189*). Most of the work published in the literature refers to amperometric biosensors as they have advantages of higher sensitivity and selectivity. The general mechanism of amperometric biosensors can be elucidated by the general reaction sequence **(Figure 5)**, which leads to an amplification of the signal (*190*, *191*). Biosensors with immobilized tyrosinase have been prepared with solid graphite (*192*, *193*) or composite electrodes such as carbon paste (*190*, *192*, *194*), epoxy/graphite (*195*), or Teflon/graphite (*196*) and hydrophobic semisolid matrices (*197*), but it was found that the solid graphite electrodes are more sensitive than the composite electrodes and can detect micromolar concentrations of phenols and catechols (*192*). Another sensor for the determination of phenols in a continuous flow system with a detection limit of 14 ppb has been developed using mushroom tyrosinase (*198*). Liu et al. (*199*) used co-immobilization of mushroom tyrosinase and the mediator, $Fe(CN)_6^{4-}$, on Al_2O_3 sol−gel membranes and reported high stability of the enzyme with a high functional activity of 5×10^{-8} M. Recently, another highly sensitive sensor for subpicomolar detection of phenols has been developed (*193*) by immobilization of mushroom tyrosinase in a positively charged chitosan (N-deacetylated derivative of chitin) film on a glassy carbon electrode, and the resulting sensor offered a high sensitivity (150 nA/nM) for monitoring phenols. A capillary membrane bioreactor, using immobilized mushroom tyrosinase for effective removal of phenols from the industrial effluents, has been prepared (*200*). To remove the colored quinone-type products of the reaction, a packed column containing chitosan was integrated into the system, and this provided a two-stage bioremediation, which resulted in almost complete removal of the colored quinones from the system. Another important industrial application of mushroom tyrosinase reported by Espín et al. (*201*) is the enzymatic synthesis of the diphenolic antioxidant hydroxytyrosol (HTyr) that mainly occurs in virgin olive oil and olive oil mill wastewater (vegetative water). This diphenol was obtained from its low-cost monophenolic precursor, tyrosol, in the presence of both tyrosinase and ascorbic acid. Although a fast chemical method is also available for HTyr synthesis, the cost of substrate is too high and the compounds obtained need further purification to get rid of any possible contamination (*202*−*204*), so the enzymatic method could be used as an alternative and nonpolluting procedure to obtain HTyr. The reaction synthesis is continuous, easy to perform, and adaptable to a bioreactor for industrial purposes.

7. SUMMARY AND CONCLUSIONS

The above studies show that mushroom tyrosinase continues to be the subject of extensive research due to its easy availability

and vast clinical and industrial importance. The role of tyrosinase in mushroom for causing browning is well established; browning occurs due to enzymatic oxidation of phenols, resulting in the mushroom's shorter shelf life and loss of nutritional value. This implies that understanding of the activators and inhibitors, which interconvert latent and active forms, is of crucial importance in finding novel and more consumer-compatible approaches toward regulation of the discoloration process. For this reason, there has been an active interest among the researchers in this field during the past three decades to identify better inhibitors for mushroom tyrosinase. To achieve this goal, different types of compounds from both natural and synthetic sources have been investigated. Obviously, more efforts are still needed in this direction so that better inhibitors can be identified. As various contradictory reports are available in the literature regarding the cause and relative extent of inhibition among various compounds, more insight is required to establish a confirmed structure and activity relationship. Furthermore, inhibitors from natural sources have a great potential in the food industry, as they are considered to be safe and largely free from adverse side effects.

Besides being used in the treatment of some dermatological disorders associated with melanin hyperpigmentation, tyrosinase inhibitors have found an important role in the cosmetic industry for their skin-whitening effect and depigmentation after sunburn. However, more concrete studies with human tyrosinase from a clinical point of view are required. Another important clinical application of mushroom tyrosinase includess its role in the treatment of vitiligo as the enzyme acts as the marker of this disease; a number of studies have been conducted on animal models, but still more research has to be done to cure vitiligo in human beings. Recent studies indicated a possible negative role of mushroom tyrosinase in the metabolism and bioactivation of agaritine and other mushroom hydrazines, in the latter case forming genotoxic metabolites,which contributes to the mutagenicity, but the metabolism or carcinogenicity of hydrazines in animals remains to be elucidated. In contrast, Shi et al. (*161*) reported that the genoprotective effect exhibited by *A. bisporus* is associated with mushroom tyrosinase, which is dependent upon enzymatic hydroxylation of tyrosine to L-dopa and subsequently to dopaquinone, but the basis of the observed effect is not yet known, so further studies are required in this field. The possible role of mushroom tyrosinase in the treatment of malignant melanoma in the form of MDEPT is established and, being a highly selective drug delivery system, it will have a promising role in the treatment of malignant melanoma. However, further in vivo research is required before a clinical trial is made. Another important aspect, which is gaining popularity, is immobilization of tyrosinase for construction of bioreactors to produce L-dopa; however, the commercial applicability of L-dopa production is yet to be established, and more research work has to be done in this exciting field. The use of mushroom tyrosinase as a biosensor as well as a catalyst for the determination and detoxification of phenolic compounds from industrial effluents is an eco-friendly method of toxic waste removal. To date, information regarding the physiological role of tyrosinase in fungi is very limited, and also the biochemical information related to kinetic characterization and the relationship between various isoforms needs further elucidation. Moreover, the X-ray crystallographic structure of tyrosine is needed, which can shed more light on the action mechanism of tyrosinase and will be helpful in in vitro mutagenesis studies including antisense RNA techniques and gene silencing, which will help to decrease production of tyrosinase in vivo.

Taken together, much more research on mushroom tyrosinase is required to find the role of this enzyme in other unexplored fields, which will be helpful in designing or improving enzymatic activities for various applications.

LITERATURE CITED

(1) Bourquelot, E.; Bertrand, A. A re-examination of the Raper's scheme: Cyclodopa as a biological precursor of eumelanin. *C. R. Soc. Biol.* **1895**, *47*, 582−584.

(2) Vanneste, W. H.; Zuberbuhler, A. Copper containing oxygenases. In *Molecular Mechanism of Oxygen Activation*; Nayaishi, O., Ed.; Academic Press: New York, 1974; pp 371−404.

(3) Lerch, K. Copper monooxygenases: Tyrosinase and dopamine β-monooxygenase. In *Metal Ions in Biological Systems*; Sigel, H., Ed.; Dekker: New York, 1981; pp 143−186.

(4) Mayer, A. M. Polyphenol oxidases in plants—Recent progress. *Phytochemistry* **1987**, *26*, 11−20.

(5) Zawistowski, J.; Biliaderis, C. G.; Eskin, N. A. M. Polyphenoloxidase. In *Oxidative Enzymes in Foods*; Robinson, D. S., Eskin, N. A. M., Eds.; Elsevier Science: London, U.K., 1991; pp 217−273.

(6) 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.

(7) Mayer, A. M.; Harel, E. Polyphenoloxidases in plants. *Phytochemistry* **1979**, *18*, 193−215.

(8) Korner, A.; Pawelek, J. Mammalian tyrosinase catalyzes three reactions in the biosynthesis of melanin. *Science* **1982**, *217*, 1163−1165.

(9) Schoot-Uiterkamp, A. J. M.; Mason, H. S. Magnetic dipole−dipole coupled Cu(II) pairs in nitric oxide-treated tyrosinase: A structural relationship between the active sites of tyrosinase and hemocyanin. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 993−996.

(10) Nishioka, K. Particulate tyrosinase of human malignant melanoma. Solubilization, purification following trypsin treatment, and characterization. *Eur. J. Biochem.* **1978**, *85*, 137−146.

(11) Sánchez-Ferrer, A.; Villalba, J.; García-Carmona, F. Triton X-114 as a tool for purifying spinach polyphenol oxidase. *Phytochemistry* **1989**, *28*, 1321−1325.

(12) Sánchez-Ferrer, A.; Bru, R.; García-Carmona, F. Partial purification of a thylakoid-bound enzyme using temperature-induced phase partitioning. *Anal. Biochem.* **1990**, *184*, 279−282.

(13) Raper, H. S. The anaerobic oxidases. *Physiol. Rev.* **1928**, *8*, 245−282.

(14) Mason, H. S. The chemistry of melanin. III. Mechanism of the oxidation of trihydroxyphenylalanine by tyrosinase. *J. Biol. Chem.* **1948**, *172*, 83−99.

(15) Lerner, A. B.; Fitzpatrick, T. B.; Calkins, E.; Summerson, W. H. Mammalian tyrosinase—preparation and properties. *J. Biol. Chem.* **1949**, *179*, 185−195.

(16) Kobayashi, T.; Vieira, W. D.; Potterf, B.; Sakai, C.; Imokawa, G. Modulation of melanogenic protein expression during the switch from eu- to phenomelanogenesis. *J. Cell Sci.* **1995**, *108*, 2301−2309.

(17) Olivares, C.; Jimenez-Cervantes, C.; Lozano, J. A.; Solano, F.; Garcia-Borron, J. C. The 5,6-dihydroxyindole-2-carboxylic acid (DHICA) oxidase activity of human tyrosinase. *Biochem. J.* **2001**, *354*, 131−139.

(18) Priestly, G. C. *Molecular Aspects of Dermatology*; Wiley: Chichester, U.K., 1993.

(19) Gadd, G. W. Melanin production and differentiation in batch cultures of the polymorphic fungus. *FEMS Microbiol. Lett.* **1980**, *9*, 237−240.

(20) Bell, A. A.; Wheeler, M. H. Biosynthesis and functions of fungal melanins. *Annu. Rev. Phytopathol.* **1986**, *24*, 411−451.

(21) Zimmerman, W. C.; Blanchette, R. A.; Burnes, T. A.; Farrel, R. L. Melanin and perithecial development in *Ophiostoma piliferum*. *Mycology* **1995**, *87*, 857−863.

(22) Martinez, M. V.; Whitaker, J. R. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **1995**, *6*, 195−200.
(23) Sánchez-Ferrer, A.; Rodríguez-López, J. N.; García-Cánovas, F.; García-Carmona, F. Tyrosinase: a comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1995**, *1247*, 1−11.
(24) Strothkemp, K. G.; Jolley, R. L.; Mason, H. S. Quaternary structure of mushroom tyrosinase. *Biochem. Biophys. Res. Commun.* **1976**, *70*, 519−524.
(25) Robb, D. A.; Gutteridge, S. Polypeptide composition of two fungal tyrosinases. *Phytochemistry* **1981**, *20*, 1481−1485.
(26) Wichers, H. J.; Gerritsen, Y. A. M.; Chapelon, C. G. J. Tyrosinase isoforms from the fruiting bodies of *Agaricus bisporus*. *Phytochemistry* **1996**, *43*, 333−337.
(27) 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. *Mushroom Sci*. **1995**, *2*, 723−728.
(28) Lang, W. H.; van Holde, K. E. Cloning and sequencing of *Octopus dofleini* hemocyanin cDNA: Derived sequences of functional units Ode and Odf. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 244−248.
(29) Jackman, M. P.; Hajnal, A.; Lerch, K. Albino mutants of *Streptomyces glaucescens* tyrosinase. *Biochem. J*. **1991**, *274*, 707−713.
(30) Himmelwright, R. S.; Eickman, N. C.; Lu Bien, C. D.; Lerch, K.; Solomon, E. I. Chemical and spectroscopic studies of the binuclear copper active site of *Neurospora* tyrosinase: Comparison to hemocyanins. *J. Am. Chem. Soc*. **1980**, *102*, 7339−7344.
(31) Beltramini, M.; Salvato, B.; Santamaria, M.; Lerch, K. The reaction of CN^- with the binuclear copper site of *Neurospora* tyrosinase: its relevance for a comparison between tyrosinase and hemocyanin active sites. *Biochim. Biophys. Acta* **1990**, *1040*, 365−372.
(32) Fenoll, L. G.; Rodríguez-López, J. N.; García-Sevilla, F.; García-Ruiz, P. A.; Varón, R.; García-Cánovas, F.; Tudela, J. Analysis and interpretation of the action mechanism of mushroom tyrosinase on monophenols and diphenols generating highly unstable *o*-quinone. *Biochim. Biophys. Acta* **2001**, *1548*, 1−22.
(33) Pomerantz, S. H.; Warner, M. C. 3,4-Dihydroxy-L-phenylalanine as the tyrosinase cofactor. Occurrence in melanoma and binding constant. *J. Biol. Chem*. **1967**, *242*, 5308−5314.
(34) Burton, S. G. Biocatalysis with polyphenol oxidase: a review. *Catal. Today* **1994**, *22*, 459−487.
(35) Ros-Martinez, J. R.; Rodríguez-López, J. N.; Castellanos, R. V.; García-Cánovas, F. Discrimination between two kinetic mechanisms for the monophenolase activity of tyrosinase. *Biochem. J*. **1993**, *294*, 621−623.
(36) Cooksey, C. J.; Garratt, P. J.; Land, E. J.; Pavel, S.; Ramsden, C. A.; Riley, P. A.; Smit, N. P. M. Evidence of the indirect formation of the catecholic intermediate substrate responsible for the autoactivation kinetics of tyrosinase. *J. Biol. Chem*. **1997**, *272*, 26226−26235.
(37) Halliwell, B. Superoxide and hydroxylation reactions. In *Superoxide and Superoxide Dismutases*; Michelson, A. M., McCord, J. M., Fridovich, I., Eds.; Academic Press: London, U.K., 1977; pp 335−349.
(38) Stüssi, H.; Rast, D. M. The biosynthesis and possible function of γ-glutaminyl-4-hydroxybenzene in *Agaricus bisporus*. *Phytochemistry* **1981**, *20*, 2347−2352.
(39) Jiménez, M.; García-Carmona, F. Hydrogen peroxide-dependent 4-*tert*-butylphenol hydroxylation by tyrosinase a new catalytic activity. *Biochem. Biophys. Acta* **1996**, *1297*, 33−39.
(40) Dawson, C. R.; Tarpley, W. B. The copper oxidases. In *The Enzymes*; Sumner, J. B., Myrbäck, K., Eds.; Academic Press: New York, 1951; pp 454−598.
(41) Zhang, X.; van Leeuwen, J.; Wichers, H. J.; Flurkey, W. H. Characterization of tyrosinase from the cap flesh of *Portabella* mushrooms. *J. Agric. Food Chem*. **1999**, *47*, 374−378.
(42) Espín, J. C.; García-Ruiz, P. A.; Tudela, J.; García-Cánovas, F. Study of stereospecificity in mushroom tyrosinase. *Biochem. J*. **1998**, *331*, 547−551.
(43) Taylor, S. L.; Bush, R. K. Sulfites as food ingredients. *Food Technol*. **1986**, *40*, 47−52.
(44) Ohyama, Y.; Mishima, Y. Melanogenesis inhibitory effects of kojic acid and its action mechanism. *Fragrance J*. **1990**, *6*, 53.
(45) Palumbo, A.; d'Ischia, M.; Misuraca, G.; Prota, G. Mechanism of inhibition of melanogenesis by hydroquinone. *Biochim. Biophys. Acta* **1991**, *1073*, 85−90.
(46) Maeda, K.; Fukuda, M. *In vitro* effectiveness of several whitening cosmetic components in human melanocytes. *J. Soc. Cosmet. Chem.* **1991**, *42*, 361−368.
(47) Kubo, I.; Yokokawa, Y. Two tyrosinase inhibiting flavonol glycosides from *Buddleia coriacea*. *Phytochemistry* **1992**, *31*, 1075−1077.
(48) Kubo, I.; Yokokawa, Y.; Kinst-Hori, I. Tyrosinase inhibitors from Bolivian medicinal plants. *J. Nat. Prod*. **1995**, *58*, 739−743.
(49) Kubo, I.; Kinst-Hori, I. Flavonols from saffron flower: tyrosinase inhibitory activity and inhibition mechanism. *J. Agric. Food Chem.* **1999**, *47*, 4121−4125.
(50) Kubo, I.; Kinst-Hori, I.; Ishiguro, K.; Chaudhuri, S. K.; Sanchez, Y.; Ogura, T. Tyrosinase inhibitory flavonoids from *Heterotheca inuloides* and their structural functions. *Bioorg. Med. Chem. Lett*. **1994**, *4*, 1443−1446.
(51) Chen, Q. X.; Kubo, I. Kinetics of mushroom tyrosinase inhibition by quercetin. *J. Agric. Food Chem.* **2002**, *50*, 4108−4112.
(52) Ha, T. J.; Yang, M. S.; Jang, D. S.; Choi, S. U.; Park, K. H. Inhibitory activities of flavanone derivatives isolated from *Sophora flavescens* for melanogenesis. *Bull. Korean Chem. Soc*. **2001**, *22*, 97−99.
(53) Kubo, I.; Kinst-Hori, I.; Chaudhuri, S. K.; Kubo, Y.; Sanchez, Y.; Ogura, T. Flavonols from *Heterotheca inuloides*: Tyrosinase inhibitory activity and structural criteria. *Bioorg. Med. Chem.* **2000**, *8*, 1749−1755.
(54) Badria, F. A.; el Gayyar, M. A. A new type of tyrosinase inhibitors from natural products as potential treatments for hyperpigmentation. *Boll. Chim. Farma.* **2001**, *140*, 267−71.
(55) No, J. K.; Soung, D. Y.; Kim, Y. J.; Shim, K. H.; Jun, Y. S.; Rhee, S. H.; Yokozawa, T.; Chung, H. Y. Inhibition of tyrosinase by green tea components. *Life Sci.* **1999**, *65*, 241−246.
(56) Kim, J. H.; Sapers, G. M.; Choi, S. W. Identification of tyrosinase inhibitor from *Galla rhois*. *Food Sci. Biotechnol*. **1998**, *7*, 56−59.
(57) Menon, S.; Fleck, R. W.; Yong, G.; Strothkamp, K. G. Benzoic acid inhibition of α, β and γ isozymes of *Agaricus bisporus* tyrosinase. *Arch. Biochem. Biophys*. **1990**, *280*, 27−30.
(58) Kermasha, S.; Goetghebeur, M.; Monfette, A.; Metchet, M.; Rovelt, M. Inhibitory effects of cysteine and aromatic acids on tyrosinase activity. *Phytochemistry* **1993**, *34*, 349−353.
(59) Kubo, I.; Kinst-Hori, I.; Kubo, Y.; Yamagiwa, Y.; Kamikawa, T.; Haraguchi, H. Molecular design of antibrowning agents. *J. Agric. Food Chem.* **2000**, *48*, 1393−1399.
(60) Kubo, I.; Kinst-Hori, I.; Yokokawa, Y. Tyrosinase inhibitors from *Anacardium occidentale* fruits. *J. Nat. Prod*. **1994**, *57*, 545−551.
(61) Lim, J. Y.; Ishiguro, K.; Kubo, I. Tyrosinase inhibitory *p*-coumaric acid from ginseng leaves. *Phytother. Res.* **1999**, *13*, 371−375.
(62) Shin, N. H.; Ryu, S. Y.; Choi, E. J.; Kang, S. H.; Chang, I. M.; Min, K. R.; Kim. Y. Oxyresveratrol as the potent inhibitor on dopa oxidase activity of mushroom tyrosinase. *Biochem. Biophys. Res. Commun.* **1998**, *243*, 801−803.
(63) Lee, S. E.; Kim, M. K.; Lee, S. G.; Ahn, Y. J.; Lee, H. S. Inhibitory effects of *Cinnamomum cassia* bark-derived materials on mushroom tyrosinase. *Food Sci. Biotechnol*. **2000**, *9*, 330−333.
(64) Kubo, I.; Kinst-Hori, I. Tyrosinase inhibitory activity of the olive oil flavor compounds. *J. Agric. Food Chem.* **1999**, *47*, 4574−4578.

(65) Kubo, I.; Kinst-Hori, I. 2-Hydroxy-4-methoxy benzaldehyde: a potent tyrosinase inhibitor from African medicinal plants. *Planta Med.* **1999**, *65*, 19−22.
(66) Kubo, I.; Kinst-Hori, I. Tyrosinase inhibitors from anise oil. *J. Agric. Food Chem.* **1998**, *46*, 1268−1271.
(67) Kubo, I.; Kinst-Hori, I. Tyrosinase inhibitors from cumin. *J. Agric. Food Chem.* **1988**, *46*, 5338−5341.
(68) Lee, H. S. Tyrosinase inhibitors of *Pulsatilla cernua* root-derived materials. *J. Agric. Food Chem.* **2002**, *50*, 1400−1403.
(69) Tanford, C. In *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*; Wiley: New York, 1980.
(70) Wilcox, D. E.; Porras, A. G.; Hwang, Y. T.; Lerch, K.; Winkler, M. E.; Solomon, E. I. Substrate analogue binding to the coupled binuclear copper active site in tyrosinase. *J. Am. Chem. Soc.* **1985**, *107*, 4015−4027.
(71) Conrad, J. S.; Dawson, S. R.; Hubbard, E. R.; Meyers, T. E.; Strothkamp, K. G. Inhibitor binding to the binuclear active site of tyrosinase: Temperature, pH, and solvent deuterium isotope effects. *Biochemistry* **1994**, *33*, 5739−5744.
(72) Jiménez, M.; Chazarra, S.; Escribano, J.; Cabanes, J.; García-Carmona, F. Competitive inhibition of mushroom tyrosinase by 4-substituted benzaldehydes. *J. Agric. Food Chem.* **2001**, *49*, 4060−4063.
(73) Jiménez, M.; García-Carmona, F. 4-Substituted resorcinols (sulfite alternatives) as slow binding inhibitors of tyrosinase catecholase activity. *J. Agric. Food Chem.* **1997**, *45*, 2061−2065.
(74) Madhosingh, C.; Sundberg, L. Purification and properties of tyrosinase inhibitor from mushroom. *FEBS Lett.* **1974**, *49*, 156−158.
(75) Goetghebeur, M.; Kermasha, S. Inhibition of polyphenol oxidase by copper-metallothionein from *Aspergillus niger*. *Phytochemistry* **1996**, *42*, 935−940.
(76) Espin, J. C.; Jolivet, S.; Wichers, H. J. Inhibition of mushroom polyphenoloxidase by Agaritine. *J. Agric. Food Chem*. **1998**, *46*, 2976−2980.
(77) Espin, J. C.; Wichers, H. J. Effect of captopril on mushroom tyrosinase activity *in vitro*. *Biochim. Biophys. Acta* **2001**, *1554*, 289−300.
(78) Andrawis, A.; Kahn, V. Effect of methimazole on the activity of mushroom tyrosinase. *Biochem. J.* **1996**, *235*, 91−96.
(79) Andrawis, A.; Kahn, V. Inactivation of mushroom tyrosinase by hydrogen peroxide. *Phytochemistry* **1985**, *24*, 397−405.
(80) Kahn, V.; Andrawis, A. Multiple effect of hydroxylamine on mushroom tyrosinase. *Phytochemistry* **1986**, *25*, 333−337.
(81) Shiino, M.; Watanabe, Y.; Umezawa, K. Synthesis of *N*-substituted *N*-nitrosohydroxylamines as inhibitors of mushroom tyrosinase. *Bioorg. Med. Chem.* **2001**, *9*, 1233−1240.
(82) Jolley, R. L., Jr.; Evans, L. H.; Makino, N.; Mason, H. S. Oxytyrosinase. *J. Biol. Chem.* **1974**, *249*, 335−345.
(83) Kahn, V.; Andrawis, A. Effect of Tiron on monohydroxy and *o*-dihydroxyphenolase activity of mushroom tyrosinase. *Phytochemistry* **1987**, *26*, 2675−2678.
(84) Lindbladh, C.; Rorsman, H.; Rosengren, E. The effect of catalase on the inactivation of tyrosinase by ascorbic acid and by cysteine or glutathione. *Acta Dermato-Venereol.* **1983**, *63*, 209−214.
(85) Schved, F.; Kahn, V. Effect of different isomers of dihydroxybenzoic acids (DBA) on the rate of DL-dopa oxidation by mushroom tyrosinase. *Pigment Cell Res*. **1992**, *5*, 58−64.
(86) Cabanes, J.; Garcia-Canovas, F.; Tudela, J.; Lozano, J. A.; García-Carmona, F. L-Mimosine, a slow-binding inhibitor of mushroom tyrosinase. *Phytochemistry* **1987**, *26*, 917−919.
(87) Cabanes, J.; Chazarra, S.; Garcia-Carmona, F. Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. *J. Pharm. Pharmacol*. **1994**, *46*, 982−985.
(88) Valero, E.; Garcia-Moreno, M.; Varon, R.; Garcia-Carmona, F. Time-dependent inhibition of grape polyphenoloxidase by tropolone. *J. Agric. Food Chem.* **1991**, *39*, 1043−1046.
(89) Kahn, V.; Andrawis, A. Inhibition of mushroom tyrosinase by tropolone. *Phytochemistry* **1985**, *24*, 905−908.
(90) Mayer, A.; Harel, E. Phenoloxidase and their significance in fruits and vegetables. In *Food Enzymology*; Fox, P. F., Ed.; Elsevier: London, U.K., 1991; pp 373−398.
(91) Morrison, J. F. The slow-binding and slow, tight-binding inhibition of enzyme-catalysed reactions. *Trends Biotechnol.* **1982**, *7*, 102−105.
(92) Pérez-Gilabert, M.; García-Carmona, F. Dimethyl sulfide, a volatile flavor constituent, is a slow-binding inhibitor of tyrosinase. *Biochem. Biophys. Res. Commun*. **2001**, *285*, 257−261.
(93) Konanayakam, M.; Sastry, S. K. Kinetics of shrinkage of mushrooms during blanching. *J. Food Sci*. **1988**, *53*, 1406−1411.
(94) Decareau, R. V. *Microwaves in Food Processing Industry*; Academic Press: London, U.K., 1985.
(95) Rodriguez-Lopez, J. N.; Fenoll, L. G.; Tudela, J.; Devece, C.; Sanchez-Hernandez, D.; de los Reyes, E.; Garcia-Canovas, F. Thermal inactivation of mushroom polyphenol oxidase employing 2450 MHz microwave radiation. *J. Am. Food Chem.* **1999**, *47*, 3028−3035.
(96) Devece, C.; Rodriguez-Lopez, J. N.; Fenoll, L. G.; Tudela, J.; Catala, J. M.; de los Reyes, E.; Garcia-Canovas, F. Enzyme inactivation analysis for industrial blanching applications: comparison of microwave, conventional, and combination heat treatments on mushroom polyphenoloxidase activity. *J. Agric. Food Chem*. **1999**, *47*, 4506−4511.
(97) Rouet-Mayer, M. A.; Philippon, J. Inhibition of catechol oxidases from apples by sodium chloride. *Phytochemistry* **1986**, *25*, 2717−2719.
(98) Janovitz-Klapp, A. H.; Richard, F. C.; Goupy, P. M.; Nicolas, J. J. Inhibition studies on apple polyphenol oxidase. *J. Agric. Food Chem.* **1990**, *38*, 926−931.
(99) Sayavecra-Soto, L. A.; Montgomery, M. W. Inhibition of polyphenoloxidase by sulfite. *J. Food Sci*. **1986**, *51*, 1531−1536.
(100) Sapers, G. M.; Ziolkowski, M. A. Comparison of erythorbic and ascorbic acids as inhibitors of enzymatic browning in apple. *J. Food Sci.* **1987**, *52*, 1732−1733.
(101) Hsu, A. F.; Shieh, J. J.; Bills, D. D.; White, K. Inhibition of mushroom polyphenoloxidase by ascorbic acid derivatives. *J. Food Sci.* **1988**, *53*, 765−767.
(102) Kahn, V. Effect of proteins, protein hydrolyzates and amino acids on *o*-dihydroxyphenolase activity of polyphenol oxidase of mushroom, avocado, and banana. *J. Food Sci.* **1985**, *50*, 111−115.
(103) Dudley, E. D.; Hotchkiss, J. H. Cysteine as an inhibitor of polyphenol oxidase. *J. Food Biochem.* **1989**, *13*, 65−75.
(104) Golan-Goldhirsch, A.; Whitaker, J. R. Effect of ascorbic acid, sodium bisulfite, and thiol compounds on mushroom polyphenol oxidase. *J. Agric. Food Chem.* **1984**, *32*, 1003−1009.
(105) Santerre, C. R.; Cash, J. N.; Vannorman, D. J. Ascorbic acid/citric acid combinations in the processing of frozen apple slices. *J. Food Sci.* **1988**, *53*, 1713−1716.
(106) Sapers, G. M.; Hicks, K. B.; Phillips, J. G.; Garzarella, L.; Pondish, D. L.: Matulaitis, R. M.; McCormack, T. J.; Sondey, S. M.; Seib, P. A.; El-Atawy, Y. S. Control of enzymatic browning in apple with ascorbic acid derivatives, polyphenol oxidase inhibitors, and complexing agents. *J. Food Sci*. **1989**, *54*, 997−1002.
(107) Taoukis, P. S.; Labuza, T. P.; Lillemo, J. H.; Lin, S. W. Inhibition of shrimp melanosis (black spot) by ficin. *Lebensm. Wiss. Technol*. **1990**, *23*, 52−54.
(108) Iyengar, R.; Boumont, C. W.; McEvily, A. 4-Hexylresorcinol and prevention of shrimp blackspots: residual analysis. *J. Food Compos. Anal.* **1991**, *4*, 148−157.
(109) McEvily, A. J.; Iyengar, R.; Otwell, W. S. Sulfite alternative prevents shrimp melanosis. *Food Technol.* **1991**, *45*, 80−86.
(110) Frankos, V. H.; Schmitt, D. F.; Haws, L. C.; McEvily, A. J.; Iyengar, R.; Miller, S. A. Generally recogniesd as safe (GRAS) evaluation of 4-hexylresorcinal for use as processing aid for prevention of melanosis in shrimp. *Regul. Toxicol. Pharmacol.* **1991**, *14*, 202−212.
(111) Burdock, G. A. *Fenaroli's Handbook of Flavor Ingredients*; CRC Press: Boca Raton, FL, 1995.

(112) Yagi, A.; Kanbara, T.; Morinobu, N. Inhibition of mushroom tyrosinase by *Aloe* extract. *Planta Med.* **1987**, *53*, 515−517.
(113) Maeda, K.; Fukuda, M. Arbutin: mechanism of its depigmenting action in human melanocyte culture. *J. Pharmacol. Exp. Ther.* **1996**, *276*, 765−769.
(114) Funyama, M.; Arakawa, H.; Yamamoto, R.; Nishino, T.; Shin, T.; Murao, S. Effects of α and *β* arbutin on the activity of tyrosinase from mushroom and mouse melanoma. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 143−144.
(115) Jin, Y. H.; Lee, S. J.; Chung, M. H.; Park, J. H.; Park, Y. I.; Cho, T. H.; Lee, S. K. Aloesin and arbutin inhibit tyrosinase activity in a synergistic manner *via* a different action mechanism. *Arch. Pharmacal Res.* **1999**, *22*, 232−236.
(116) 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.
(117) Jiménez, M.; García-Carmona, F. The effect of sodium dodecyl sulphate on polyphenol oxidase. *Phytochemistry* **1996**, *42*, 1503−1509.
(118) Nellaiappan, K.; Sugumaran, M. On the presence of prophenoloxidase in the hemolymph of the horseshoe crab *Limulus*. *Comp. Biochem. Physiol.* **1996**, *113B*, 163−168.
(119) Chazarra, S.; Cabanes, J.; Escribano, J.; García-Carmona, F. Kinetic study of the suicide inactivation of latent polyphenoloxidase from iceberg lettuce (*Lactuca sativa*) induced by 4-*tert*-butylcatechol in the presence of SDS. *Biochem. Biophys. Acta* **1997**, *1339*, 297−303.
(120) Escribano, J.; Cabanes, J.; García-Carmona, F. Characterization of latent polyphenoloxidase in table beet: effect of sodium dodecyl sulfate. *J. Sci. Food Agric.* **1997**, *73*, 34−38.
(121) Jiménez-Atienzar, M.; Angeles-Pedreno, M.; García-Carmona, F. Activation of polyphenol oxidase by polyamines. *Biochem. Int.* **1991**, *25*, 861−868.
(122) Fujita, Y.; Uraga, Y.; Ichisima, E. Molecular cloning and nucleotide sequence of the protyrosinase gene, melO, from *Aspergillus oryzae* and expression of the gene in yeast cells. *Biochim. Biophys. Acta* **1995**, *1261*, 151−154.
(123) 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.
(124) Jiménez-Cervantes, C.; García-Borrón, J. C.; Lozano, J. A.; Solano, F. Effect of detergents and endogenous lipids on the activity and properties of tyrosinase and its related proteins. *Biochim. Biophys. Acta* **1995**, *1243*, 421−430.
(125) Asada, N.; Fukumitsu, T.; Fujimoto, K.; Masuda, K. Activation of prophenoloxidase with 2-propanol and other organic compounds in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **1993**, *23*, 515−520.
(126) 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.
(127) Burton, K. S.; Love, M. E.; Smith, J. F. Biochemical changes associated with mushroom quality in *Agaricus* spp. *Enzyme Microb. Technol.* **1993**, *15*, 736−741.
(128) 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.
(129) 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.
(130) Espín, J. C.; Wichers, H. J. Activation of a latent mushroom (*Agaricus bisporus*) tyrosinase isoform by sodium dodecyl sulfate (SDS). Kinetic properties of the SDS-activated isoform. *J. Agric. Food Chem.* **1999**, *47*, 3518−3525.
(131) Espín, J. C.; Wichers, H. J. Kinetics of activation of latent mushroom (*Agaricus bisporus*) tyrosinase by benzyl alcohol. *J. Agric. Food Chem.* **1999**, *47*, 3503−3508.
(132) Espín, J. C.; van Leeuwen, J.; Wichers, H. J. Kinetic study of the activation process of a latent mushroom (*Agaricus bisporus*) tyrosinase by serine proteases. *J. Agric. Food Chem.* **1999**, *47*, 3509−3517.
(133) Rainey, P. B.; Brodey, C. L.; Johnstone, K. Biological properties and spectrum of activity of tolassin, a lipodepsipeptide toxin produced by the mushroom pathogen *Pseudomonas tolaasii*. *Physiol. Mol. Plant Pathol.* **1991**, *39*, 57−70.
(134) Rescigno, A.; Sanjust, E.; Soddu, G.; Rinaldi, A. C.; Sollai, F.; Curreli, N.; Rinaldi, A. Effect of 3-hydroxyanthranilic acid on mushroom tyrosinase activity. *Biochim. Biophys. Acta* **1998**, *1384*, 268−276.
(135) Baharav, E.; Merimski, O.; Shoenfeld, Y.; Zigelman, R.; Gilbrud, B.; Yecheskel, G.; Youinou, P.; Fishman, P. Tyrosinase as an autoantigen in patients with vitiligo. *Clin. Exp. Immunol.* **1996**, *105*, 84−88.
(136) Zehtab, T.; Yazdanparast, R.; Rafiei, S. Inhibition of experimental autoimmune vitiligo by oral administration of mushroom tyrosinase. *Cytobios* **2001**, *105*, 27−34.
(137) Kemp, E. H.; Gawkrodger, D. J.; MacNeil, S.; Watson, P. F.; Weetman, A. P. Detection of tyrosinase autoantibodies in patients with vitiligo using ^{35}S-labeled recombinant human tyrosinase in a radioimmunoassay. *J. Invest. Dermatol.* **1997**, *109*, 69−73.
(138) Fishman, P.; Merimski, O.; Baharav, E.; Shoenfeld, Y. Autoantibodies to tyrosinase: the bridge between melanoma and vitiligo. *Cancer* **1997**, *79*, 1461−1464.
(139) Vogel, F. S.; Kemper, L. A.; Jeffs, P. W.; Cass, M. W.; Graham, D. G. *γ*-L-Glutaminyl-4-hydroxybenzene, an inducer of cryptobiosis in *Agaricus bisporus* and a source of specific metabolic inhibitors for melanogenic cells. *Cancer Res.* **1977**, *37*, 1133−1136.
(140) Wick, M. M.; Rosowsky, A.; Ratliff, J. Antitumor effects of L-glutamic acid dihydroxyanilides against experimental melanoma. *J. Invest. Dermatol.* **1980**, *74*, 112−114.
(141) Papaparaskeva-Petrides, C.; Ioannides, C.; Walker, R. Contribution of phenolic and quinonoid structures in the mutagenicity of the edible mushroom. *Food Chem. Toxicol.* **1993**, *31*, 561−567.
(142) Walton, K.; Walker, R.; Ioannides, C. Effect of baking and freeze-drying on the direct and indirect mutagenicity of the extracts from the edible mushroom *Agaricus bisporus*. *Food Chem. Toxicol.* **1998**, *36*, 315−320.
(143) Toth, B. Toxicities of hydrazines: A review. *In Vivo* **1988**, *2*, 209−242.
(144) Toth, B. Mushroom toxins and cancer (review). *Int. J. Oncol.* **1995**, *6*, 137−145.
(145) Walton, K.; Coombs, M. M.; Walker, R.; Ioannides, C. Bioactivation of mushroom hydrazines to mutagenic products by mammalian and fungal enzymes. *Mutat. Res.* **1997**, *381*, 131−139.
(146) Walton, K.; Coombs, M. M.; Walker, R.; Ioannides, C. The metabolism and bioactivation of agaritine and of other mushroom hydrazines by whole mushroom homogenate and by mushroom tyrosinase. *Toxicology* **2001**, *161*, 165−177.
(147) Papaparaskeva, C.; Ioannides, C.; Walker, R. Agaritine does not mediate the mutagenicity of the edible mushroom *Agaricus bisporus*. *Mutagenesis* **1991**, *6*, 213−217.
(148) Prota, G.; d'Ischia, M.; Mascagna, D. Melanogenesis as a targating strategy against metastatic melanoma—a reassessment. *Melanoma Res.* **1994**, *4*, 351−358.
(149) Valmori, D.; Pittet, M. J.; Rimoldi, D.; Liénard, D.; Dunbar, R.; Cerundolo, V.; Lejeune, F.; Cerottini, J. C.; Romero, P. An antigen-targeted approach to adoptive transfer therapy of cancer. *Cancer Res.* **1999**, *59*, 2167−2173.
(150) Jordan, A. M.; Khan, T. H.; Osborn, H. M. I.; Photiou, A.; Riley, P. A. Melanocyte directed enzyme prodrug therapy (MDEPT): development of a targeted treatment for malignant melanoma. *Bioorg. Med. Chem.* **1999**, *7*, 1775−1780.

(151) Jordan, A. M.; Khan, T. H.; Malkin, H.; Osborn, H. M. I.; Photiou, A.; Riley, P. A. Melanocyte-directed enzyme prodrug therapy (MDEPT): Development of second generation prodrugs for targeted treatment of malignant melanoma. *Bioorg. Med. Chem*. **2001**, *9*, 1549−1558.

(152) Lougestay-Madec, R.; Florent, J. C.; Monneret, C.; Nemati, F.; Poupon, M. F. Synthesis of self-immolative glucuronide-based prodrugs of a phenol mustard. *Anti-Cancer Drug Des*. **1998**, *13*, 995−1007.

(153) McClean, S.; Costelloe, C.; Denny, W. A.; Searcey, M.; Wakelin, L. P. G. Sequence selectivity, cross-linking efficiency and cytotoxicity of DNA-targeted 4-anilinoquinoline aniline mustards. *Anti-Cancer Drug Des*. **1998**, *14*, 187−204.

(154) Lowenberg, B.; Suciu, S.; Archimbaud, E.; Haak, H.; Stryckmans, P.; de Cataldo, R.; Dekker, A. W.; Berneman, Z. N.; Thyss, A.; van der Lelie, J.; Sonneveld, P.; Visani, G.; Fillet, G.; Hayat, M.; Hagemeijer, A.; Solbu, G.; Zittoun, R. Mitoxantrone versus daunorubicin in induction-consolidation chemotherapy—the value of low-dose cytarabine for maintenance of remission, and an assessment of prognostic factors in acute myeloid leukemia in the elderly: final report. European Organization for the Research and Treatment of Cancer and the Dutch-Belgian Hemato-Oncology Cooperative Hovon Group. *J. Clin. Oncol*. **1998**, *16*, 872−881.

(155) Ourth, D. D.; Renis, H. E. Antiviral melanization reaction of *Heliothis virescens* hemolymph against DNA and RNA viruses *in vitro*. *Comp. Biochem. Physiol.* **1993**, *105*, 719−723.

(156) Ames, B. N.; Shigenaga, M. K.; Hagen, T. M. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7915−7922.

(157) Remacle, J.; Raes, M.; Toussaint, O.; Renard, P.; Rao, G. Low levels of reactive oxygen species as modulators of cell function. *Mutat. Res.* **1995**, *316*, 103−122.

(158) Halliwell, B.; Gutteridge, J. M. C. In *Free Radicals in Biology and Medicine*; Oxford University Press: Oxford, U.K., 1999.

(159) Halliwell, B.; Gutteridge, J. M. C. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **1984**, *219*, 1−14.

(160) Shi, Y. L.; James, A. E.; Benzie, I. F. F.; Buswell, J. A. Mushroom-derived preparations in the prevention of H_2O_2-induced oxidative damage to cellular DNA. *Teratog. Carcinog. Mutagen.* **2002**, *22*, 103−111.

(161) Shi, Y. L.; Benzie, I. F. F.; Buswell, J. A. Role of tyrosinase in the genoprotective effect of the edible mushroom, *Agaricus bisporus*. *Life Sci.* **2002**, *70*, 1595−1608.

(162) Gerritsen, Y. A. M.; Chapelon, C. G. J.; Wichers, H. J. The low-isoelectric point tyrosinase of *Agaricus bisporus* may be a glycoprotein. *Phytochemistry* **1994**, *35*, 573−577.

(163) Graham, D. G. Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Mol. Pharmacol.* **1978**, *14*, 633−643.

(164) Cohen, G. Oxidative stress in the nervous system. In *Oxidative Stress*; Sies, H., Ed.; Academic Press: London, U.K., 1985; pp 383−401.

(165) Husain, S.; Hadi, S. M. Strand scission in DNA induced by L-DOPA in the presence of Cu(II). *FEBS Lett.* **1995**, *364*, 75−78.

(166) Morin, B.; Davies, M. J.; Dean, R. T. The protein oxidation product 3,4-dihydroxyphenylalanine (DOPA) mediates oxidative DNA damage. *Biochem. J.* **1998**, *330*, 1059−1067.

(167) Mytilineou, C.; Han, S. K.; Cohen, G. Toxic and protective effects of L-DOPA on mesencephalic cell cultures. *J. Neurochem.* **1993**, *61*, 1470−1478.

(168) Shi, M. M.; Iwamoto, T.; Forman, H. J. γ-Glutamylcysteine synthetase and GSH increase in quinone-induced oxidative stress in BPAEC. *Am. J. Physiol.* **1994**, *267*, L414−L421.

(169) Han, S. K.; Mytilineou, C.; Cohen, G. L-DOPA up-regulates glutathione and protects mesencephalic cultures against oxidative stress. *J. Neurochem.* **1996**, *66*, 501−510.

(170) Klibanov, A. M. Immobilized enzymes and cells as practical catalysts. *Science* **1983**, *219*, 722−727.

(171) Wykes, J. R.; Dunill, P.; Lilly, M. D. Conversion of tyrosine to L-dihydroxy phenylalanine using immobilized tyrosinase. *Nat. New Biol.* **1971**, *230*, 187−191.

(172) Letts, D.; Chase, J. Chemical modification of mushroom tyrosinase for stabilization to reaction inactivation. *Adv. Exp. Med. Biol*. **1974**, *4*, 317−328.

(173) Schiller, J. G.; Liu, C. C. Immobilization of tyrosinase within polyacrylamide gel. *Biotechnol. Bioeng.* **1976**, *18*, 1405−1412.

(174) Danise, B.; Vetromile, A.; Prota, G. Immobilization of mushroom tyrosinase on activated CH-Sepharose 4B. *Atti Accad. Naz. Lincei, Cl. Sci., Fis., Mat. Nat.* **1980**, *68*, 603.

(175) Vilanova, E.; Manjon, A.; Iborra, J. L. Tyrosine hydroxylase activity of immobilized tyrosinase on Enzacryl-aa and CPG-aa supports: stabilization and properties. *Biotechnol. Bioeng.* **1984**, *26*, 1306−1312.

(176) Palmieri, G.; Giardina, P.; Desiderio, B.; Morzullo, L.; Giamberini, M.; Sannia, G. A new enzyme immobilization procedure using copper alginate gel: application to a fungal phenol oxidase. *Enzyme Microb. Technol*. **1994**, *16*, 151−158.

(177) Pialis, P.; Hamann, M. C. J.; Saville, B. A. L-DOPA production from tyrosinase immobilized on nylon 6,6. *Biotechnol. Bioeng.* **1996**, *51*, 141−147.

(178) Naidja, A.; Huang, P. M.; Bollag, J. M. Activity of tyrosinase immobilized on hydroxyaluminum-montmorillonite complexes. *J. Mol. Catal.* **1997**, *115*, 305−316.

(179) Carvalho, G. M.; Alves, T. L.; Freire, D. M. L-dopa production by immobilized tyrosinase. *Appl. Biochem. Biotechnol*. **2000**, *84*−*86*, 791−800.

(180) Munjal, N.; Sawhney, S. K. Stability and properties of mushroom tyrosinase entrapped in alginate, polyacrylamide and gelatin gels. *Enzyme Microb. Technol*. **2002**, *30*, 613−619.

(181) Atlow, S. T.; Bonadonna-Aparo, L.; Klibanov, A. M. Dephenolization of industrial waste waters catalised by polyphenol oxidase. *Biotechnol. Bioeng*. **1984**, *26*, 599−603.

(182) Ikehata, K.; Nicell, J. A. Characterization of tyrosinase for the treatment of aqueous phenols. *Bioresour. Technol*. **2000**, *74*, 191−199.

(183) Ikehata, K.; Nicell, J. A. Color and toxicity removal following tyrosinase-catalysed oxidation of phenols. *Biotechnol. Prog*. **2000**, *16*, 533−540.

(184) Morpurgo, M.; Schiavon, O.; Caliceti, P.; Veronese, F. M. Covalent modification of mushroom tyrosinase with different amphiphic polymers for pharmaceutical and biocatalysis applications. *Appl. Biochem. Biotechnol.* **1996**, *56*, 59−72.

(185) Wada S.; Ichikawa, H.; Tatsumi, K. Removal of phenols and aromatic amines from wastewater by a combination treatment with tyrosinase and a coagulant. *Biotechnol. Bioeng.* **1995**, *45*, 304−309.

(186) Wada, S.; Ichikawa, H.; Tatsumi, K. Removal of phenols from wastewater by soluble and immobilized tyrosinase. *Biotechnol. Bioeng.* **1993**, *42*, 854−858.

(187) Sun, W. Q.; Payne, G. F.; Moas, M. S. G. L.; Chu, J. H.; Wallance, K. K. Tyrosinase reaction/chitosan adsorption for removing phenols from wastewater. *Biotechnol. Prog.* **1992**, *8*, 179−186.

(188) Marko-Varga, J. G.; Emnéus, J.; Gorton, L.; Ruzgas, T. Development of enzyme-based amperometric sensors for the determination of phenolic compounds. *Trends Anal. Chem*. **1995**, *14*, 319−328.

(189) Nistor, C.; Emnéus, J.; Gorton, L.; Ciucu, A. Improved stability and altered selectivity of tyrosinase based graphite electrodes for detection of phenolic compounds. *Anal. Chim. Acta* **1999**, *387*, 309−326.

(190) Ortega, F.; Domínguez, E.; Burestedt, E.; Emnéus, J.; Gorton L.; Marko-Varga, G. Phenol oxidase-based biosensors as selective detection units in column liquid chromatography for the determination of phenolic compounds. *J. Chromatogr. A* **1994**, *675*, 65−78.

(191) Pulg, D.; Barcelo, D. Determination of phenolic compounds in water and wastewater. *Trends Anal. Chem*. **1996**, *15*, 362−375.

(192) Lutz, M.; Burestedt, E.; Emnéus J.; Lidén, H.; Gobhadi, S.; Gorton, L.; Marko-Varga, G. Effects of different additives on a tyrosinase based carbon paste electrode. *Anal. Chim. Acta* **1995**, *305*, 8−17.
(193) Wang, G.; Xu, J. J.; Ye, L. H.; Zhu, J. J.; Chen, H. Y. Highly sensitive sensors based on the immobilization of tyrosinase in chitosan. *Bioelectrochemistry* **2002**, *57*, 33−38.
(194) Moressi, M. B.; Zon, A.; Fernández, H.; Rivas, G.; Solis, V. Amperometric quantification of *Alternaria* mycotoxins with a mushroom tyrosinase modified carbon paste electrode. *Electrochem. Commun*. **1999**, *1*, 472−476.
(195) Wang, J.; Reviejo, J.; Agnes, L. Graphite-Teflon enzyme electrode. *Electroanalysis* **1993**, *5*, 575−579.
(196) Wang, J.; Varughese, K. Polishable and robust biological electrode surfaces. *Anal. Chem.* **1990**, *62*, 318−320.
(197) Liu, J.; Wang, J. Remarkable thermostability of bioelectrodes based on enzymes immobilized within hydrophobic semi-solid matrices. *Biotechnol. Appl. Biochem*. **1999**, *30*, 177−183.
(198) Bonakdar, M.; Vilchez, J. L.; Mottola, H. A. Bioamperometric sensors for phenol based on carbon paste electrodes. *J. Electroanal. Chem*. **1989**, *266*, 47−55.
(199) Liu, Z.; Deng, J.; Li, D. A new tyrosinase biosensor based on tailoring the porosity of Al_2O_3 sol−gel to co-immobilize tyrosinase and the mediator. *Anal. Chim. Acta* **2000**, *407*, 87−96.
(200) Edwards, W.; Bownes, R.; Leukes, W. D.; Jacobs, E. P.; Sanderson, R.; Rose, P. D.; Burton, S. G. A capillary membrane bioreactor using immobilized polyphenol oxidase for the removal of phenols from industrial effluents. *Enzyme Microb. Technol*. **1998**, *24*, 209−217.
(201) Espín, J. C.; Soler-Rivas, C.; Cantos, E.; Tomás-Barberán, F. A.; Wichers, H. J. Synthesis of the antioxidant hydroxytyrosol using tyrosinase as biocatalyst. *J. Agric. Food Chem*. **2001**, *49*, 1187−1193.
(202) Garcia, P.; Romero, C.; Brenes, M.; Garrido, A. Effect of metal cations on the chemical oxidation of olive *o*-diphenols in model systems. *J. Agric. Food Chem*. **1996**, *44*, 2101−2105.
(203) Bai, C.; Yan, X.; Takenaka, M.; Sekiya, K.; Nagata, T. Determination of synthetic hydroxytyrosol in rat plasma by GC-MS. *J. Agric. Food Chem*. **1998**, *46*, 3998−4001.
(204) Capasso, R.; Evidente, A.; Avolio, S.; Solla, F. A highly convenient synthesis of hydroxytyrosol and its recovery from agricultural waste waters. *J. Agric. Food Chem*. **1999**, *47*, 1745−1748.

Received for review July 26, 2002. Revised manuscript received December 13, 2002. Accepted February 6, 2003. We are grateful to the Ministry of Health and Welfare and RRC/NMR, Korea, for financial assistance.

JF020826F