Characterization of Tyrosinase from the Cap Flesh of Portabella Mushrooms

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Tyrosinase, purified from the cap flesh tissue of portabella mushrooms, was characterized with regard to its physical and biochemical properties. A native molecular size of 41 kDa for the enzyme was obtained by size exclusion chromatography, whereas SDS−PAGE indicated that the enzyme contained a single subunit with a size of ∼48 kDa under reduced and nonreduced conditions. The purified enzyme showed a single immunological cross-reacting protein after Western blotting when probed with antibodies against *Agaricus bisporus* tyrosinase. Isoelectric focusing demonstrated that the enzyme preparation, apparently homogeneous by electrophoresis, still contained three isoforms of p*I* 5.1, 5.2, and 5.3. The purified enzyme was able to oxidize a variety of mono-, di-, and triphenolic compounds. An apparent K_m of 5 mM was obtained using catechol as the substrate, and an apparent K_m of 9 mM was found using L-Dopa as a substrate. Ascorbic acid, kojic acid, tropolone, mercaptobenzothiazole, and salicylhydroxamic acid inhibited the enzyme severely at 100 μM.

Keywords: *Tyrosinase; isoforms; characterization; mushrooms; portabella*

INTRODUCTION

Several species and strains of cultivated mushrooms are available for commercial and consumer use. The common button mushroom, *Agaricus bisporus*, is produced in large quantities in the United States and Europe for use in a variety of consumer products. Other species and strains available for consumption include oyster, shiitake, enoki, morel, and crimini mushrooms. Crimini mushrooms are a brown strain of *A. bisporus* and are sometimes referred to as portabella mushrooms. Mushroom quality in cultivated mushrooms is related to whiteness, flavor, texture, and freshness. Minimally processed common button mushrooms have a shelf life that is limited to 3−4 days. During development and processing, browning of the cap and cap flesh may occur. Enzymatic browning in this type of mushroom occurs due to oxidation of phenolic compounds by tyrosinase (EC 1.14.18.1; tyrosine, L-Dopa:oxygen oxidoreductase; catecholase, diphenol oxidase, polyphenol oxidase) when cells are broken or damaged. This breakage or damage may occur during or after senescence, handling, bruising, bacterial infestations, and washing procedures. As a result, the organoleptic properties of mushrooms, such as color and taste, can be altered. In many cases, prevention and control of enzymatic browning increases consumer appeal and the nutritive value of the product.

Several tyrosinase isoforms, differing in their isoelectric points, have been identified in *A. bisporus*. Gerritsen et al. (1994) isolated a low isoelectric point tyrosinase isoform (p*I* 4.5) that was glycosylated. Two tyrosinase isoforms with p*I* values of 5.1 and 5.2 were isolated by Wichers et al. (1996) from *A. bisporus*. These latter isoforms were composed of a single subunit with a molecular size of 43 kDa and a native molecular size of 47 kDa. These monomeric enzymes exhibited both cresolase and catecholase activity associated with tyrosinase. In contrast, Papa et al. (1994a,b) isolated low IEF forms (p*I* 4.1 and 4.3) from *A. bisporus* that were associated with cresolase and catecholase activity and also isolated higher IEF forms (p*I* 4.7 and 4.9) that displayed only catecholase activity. Cloning of *A. bisporus* tyrosinase has indicated a size of 63.9 kDa for the proenzyme and a potential size of 43 kDa for the mature enzyme (Wichers et al., 1995; van Gelder et al., 1997).

Commercially available sliced portabella mushrooms contain large amounts of white cap flesh material that is susceptible to enzymatic browning. Recently, we showed that at least 10 isoforms were present in crude extracts of portabella mushrooms by analytical isoelectric focusing (IEF) (Zhang and Flurkey, 1997). The distribution of isoforms was dependent upon which tissues were examined and how well the tissues were separated from another. These studies also suggested that the tyrosinases in different tissues had different substrate and inhibitor specificities. To understand more fully the enzyme involved in this potential browning problem, we purified the tyrosinase from cap flesh material (Zhang and Flurkey, 1999). In his study, we report some of the characteristics associated with the dominant tyrosinase isoforms in portabella cap flesh.

MATERIALS AND METHODS

Enzyme Source. Sliced portabella mushrooms (Phillips Mushrooms Farms, Kennet Square, PA) were purchased from local groceries. The cap skin, gill, and stalk tissues were removed, and only the interior region of the cap flesh material, excluding the skin and gill tissue, was used for purification of the tyrosinase.

Enzyme Purification. Cap flesh portabella tyrosinase was purified as described by Zhang and Flurkey (1999). Briefly,

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crude extracts were prepared and passed through a large DEAE-cellulose column (5 × 15 cm) previously equilibrated in 100 mM phosphate buffer (pH 7.0) containing 10% glycerol (buffer A). Enzyme was eluted from the column in buffer A containing 150 mM NaCl. Selected fractions were pooled, dialyzed against buffer A, and applied to hydroxylapatite (HA) columns (1.5 × 16 cm) equilibrated in buffer A. Tyrosinase was eluted with a 10−200 mM phosphate buffer (pH 7.0) gradient, and major peak fractions were pooled. Only the p*I* 5.1−5.3 isoforms were characterized further in this study.

Spectrophotometric Enzyme Assays. Tyrosinase activity was monitored at 410 nm using catechol as the substrate and at 475 nm using 3,4-dihydroxyphenylalanine (Dopa) as the substrate. Routine assays contained 15 mM catechol or 5 mM Dopa in 3 mL of 100 mM phosphate buffer (pH 7.0). Initial rates were calculated from the linear portion of the absorbance versus time curves. One unit of activity was defined as a change in one absorbance unit per minute under these conditions. Tyrosinase activity was also assayed using 5 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH) with either catechol or Dopa as substrates as described by Winder and Harris (1991) and Espin et al. (1997) at pH 7.0 in 100 mM phosphate buffer.

pH Optima. Determination of the pH optimum was carried out in 100 mM citrate buffers ranging in pH from 2.5 to 6.0 and in 100 mM phosphate buffers ranging in pH from 6.0 to 8.0 using 15 mM catechol as the substrate.

Substrate Specificity. Tyrosinase activity was monitored spectrophotometrically using the substrates catechol, L-Dopa, D-Dopa, D,L-Dopa, chlorogenic acid, catechin, pyrogallol, tyrosine, and *p*-cresol as described earlier (Ratcliff et al., 1994; Flurkey et al., 1995; Zhang and Flurkey, 1997).

Inhibitor Studies. Inhibition studies were carried out using 15 mM catechol as the substrate in 100 mM phosphate buffer (pH 7.0). Purified enzyme was incubated with different inhibitor concentrations ranging from 10 to 1000 μM for 5 min. The mixture was then added to the substrate solution containing the same concentration of inhibitor for assay of enzyme activity. Control samples contained all of the above components except enzyme. Potential inhibitors investigated in this experiment were *o*-, *m*-, and *p*-coumaric acid, oxalic acid, *o*- and *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, pimelic acid, kojic acid, resorcinol, 4-hexylresorcinol, salicylhydroxamic acid (SHAM), orcinol, tropolone, glutathione, methimazole, sorbic acid, thiourea, cysteine, caffeic acid, phenylacetic acid, 2-mercaptobenzothiazole, phenyl salicylate, γ-aminobutyric acid, anisidine hydrochloride, phenylpyruvic acid, EDTA, 2,3-dihydroxynaphthalene, L-mimosine, L-ascorbic acid, *o*-phenanthroline, hydroxylamine, diethyldithiocarbamic acid (DIECA), NADPH, and NADH.

Kinetic Data Analysis. Catechol concentrations ranged from 0.5 to 10 mM, and Dopa concentrations ranged from 0.5 to 15 mM. Kinetic constants were determined using Enzpack 3 software (Biosoft, Cambridge, U.K.) by analysis of duplicate measurements of initial rates versus [S] data using nonlinear regression analysis.

Electrophoresis. Native polyacrylamide gel electrophoresis (PAGE) was carried out in 5% polyacrylamide gels following the method of Laemmli (1970) without the inclusion of SDS as described by Angleton and Flurkey (1984) and Zhang and Flurkey (1997). After electrophoresis, the gels were incubated in 100 mM phosphate buffer (pH 7.0) for 5−10 min followed by incubation in buffer containing 2 mM catechol. Photo contact prints were made using Kodak EDF film as soon as staining became visible. SDS−PAGE electrophoresis was carried out according to the method of Laemmli (1970) in 10% polyacrylamide gels or using the NUPAGE system (Novex, San Diego, CA). Protein molecular weight marker standards were obtained from Novex (San Diego, CA) and from Sigma Chemical Co. (St. Louis, MO). Proteins were located by staining with Coomassie Blue R-250. After destaining, photo contact prints were made using Kodak EDP films. SDS−PAGE was carried out in the presence and absence of 100 mM DTE as a reducing agent.

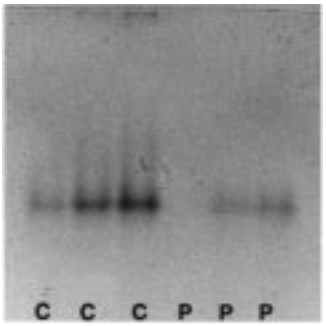

Figure 1. Native electrophoresis of crude and purified tyrosinase. Crude extracts (C; 5, 10, and 15 μL; left to right) and purified tyrosinase (P; 0.5, 1, and 1.5 μL; left to right) were subjected to native electrophoresis in 5% polyacrylamide gels. Tyrosinse activity was located by incubating gels in 0.1 M phosphate (pH 7.0) containing 2 mM L-Dopa.

Western Blotting. After SDS−PAGE, proteins were electroblotted onto PVDF membranes in 25 mM Tris, 200 mM glycine, and 20% methanol (v/v) (pH 8.6) for 1 h at 100 V. The membranes were washed in tris-buffered saline (pH 7.5, TBS) and blocked overnight at 4 °C in TBS supplemented with 3% defatted milk powder. Membranes were washed twice in TBS supplemented with 0.05% Tween 20 (TTBS) and then incubated for 2 h in a 1:1000 dilution of mouse polyclonal antibodies raised against 43 kDa tyrosinase isoforms purified earlier (Wichers et al., 1996). Blots were washed twice with TTBS and incubated for 2 h in a 1:30000 dilution of goat anti-mouse antibodies conjugated to alkaline phosphatase. The blots were washed twice and stained for alkaline phosphatase using 4-nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP).

Isoelectric Focusing. Precast agarose isoelectric focusing gels (pH 3−7) obtained from FMC Inc. (Rockland, ME) were prefocused at 10 °C for 10 min at 5 W. Enzyme samples and protein IEF markers (Bio-Rad Laboratories, Richmond, CA; Pharmacia Biotech, Piscataway, NJ; Sigma Chemical Co., St. Louis, MO) were loaded onto the gels using applicator strips or paper tabs. Focusing was carried out at 10 °C for 30 min at 5 W and liquid blotted from the gel surface. Focusing was continued until sharp bands of blue phycocyanin (p*I* 4.55, 4.65, and 4.75) became apparent. The gels were stained for enzyme by coating the surface with 2 mM catechol or 2 mM Dopa in 100 mM phosphate buffer (pH 7.0). Photo contact prints were made periodically as soon as bands appeared. Lanes containing protein IEF markers were fixed in 10% trichloroacetic acid, incubated in water overnight, stained with Coomassie blue R-250, and then destained.

Native Molecular Weight Determination. A Sephadex G-100 column (1 × 49 cm) was equilibrated with 100 mM phosphate buffer (pH 7.0). Molecular weight standards (ferritin, alcohol dehydrogenase, bovine serum albumin, ovalbumin, carbonic anhydrase, chymotrypsinogen, ribonuclease, and vitamin B12) were purchased from SigmaChemical Co. and Pharmacia Biotech and monitored by their absorbance at 280 nm. Natural logarithms of the molecular weights of the standards and tyrosinase were plotted against distance, time, volume, or V_e/V_0 (elution volume/void volume).

RESULTS AND DISCUSSION

Tyrosinase Isoforms in Portabella Mushrooms. Like many other fungal species, a variety of tyrosinase isoforms were found in portabella mushrooms. Only one tyrosinase form was identified by electrophoresis in the cap skin, cap flesh, gill, and stalk tissue from portabella mushrooms (Zhang and Flurkey, 1997). This same form was present in the crude cap flesh extracts and in the enzyme purified from cap flesh (Figure 1). At least 10 forms have been identified in crude extracts of these same tissues by IEF (Zhang and Flurkey, 1997), whereas

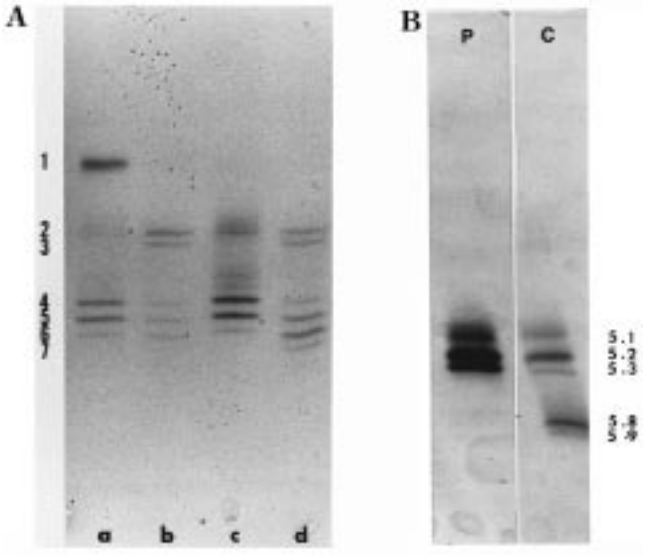

Figure 2. IEF of tyrosinase from different portabella mushroom tissuess: (A) Crude extracts from cap skin (a), cap flesh (b), gill (c), and stalk tissue (d) were subjected to IEF in pH 3−7 gradients. Tyrosinase activity was located by staining gels with 2 mM catechol in 0.1 M phosphate buffer (pH 7.0). Numbers refer to most intense isoforms visible on photographs. (B) Crude extracts from cap flesh (C) and purified cap flesh tyrosinase (P) were subjected to IEF in pH 3−7 gradients. Tyrosinase activity was located as indicated above. Numbers refer to the p*I* of isoforms determined from comparisons to known p*I* protein standards.

at least five forms were identified in crude extracts of *A. bisporus* (Wichers et al., 1996). Depending on how well the tissues are separated from one another, we have also identified similar isoforms in the cap flesh, cap skin, gill, and stalk tissue in this paper (Figure 2A). Crude cap flesh extracts contained at least five isoforms with p*I* values ranging from pH 5 to pH 6 (Figure 2A, lane b). Two of these forms with p*I* values from 5 to 5.3 comprise the major staining bands in IEF of cap flesh extracts. Purification of these forms from cap flesh tissue resulted in the isolation of an enzyme preparation that contained one isoform identified by native electrophoresis (Figure 1), but two major forms of p*I* 5.2 and 5.3 along with a minor form of p*I* 5.1 (Figure 2B) by IEF. Thus, what appears to be a homogeneous enzyme preparation when analyzed by electrophoresis is still composed of a mixture of distinct forms with slightly different p*I* values. This suggests that the isoforms are of similar size but differ in charge. Similar findings have been reported for broad bean tyrosinase (Ganesa et al., 1991).

Purification of p*I* 5.1−5.3 Tyrosinase Isoforms. The isoforms isolated in this study from portabella mushrooms appear to be similar to those isolated in *A. bisporus*. Wichers et al. (1996) isolated two tyrosinase isoforms by chromatography on HA and SEC chromatography on Sephadex G-200, followed by two consecutive passes through Mono Q columns. One of their isoforms, Ab1, appeared to consist of a single isoform with a p*I* of 5.1. A second isoform fraction, Ab2, appeared to consist of two isoforms with p*I* values of 5.1 (minor form) and 5.2 (major form). In terms of recovered protein, our yields were similar to those obtained by Wichers et al. (1996). Although our yield of enzyme activity recovered was greater than that of Wichers et al. (1996), the overall purification fold (20−25) was lower.

Papa et al. (1994a,b) were able to separate *A. bisporus* tyrosinase isoforms using a modification of the method of Nelson and Mason (1970) followed by preparative IEF. These isolated IEF forms were characterized with p*I* values of 4.1, 4.3, 4.7, and 4.9. These p*I* values are lower than those described in this paper and those of Wichers et al. (1996); however, Papa et al. (1994a,b) used mushrooms from an earlier developmental stage. Six tyrosinase isoforms, with p*I* values of 4.3, 4.4, 4.5, 4.5, 4.6, and 4.7, were isolated from browned gill tissue of *Lentinus edodes* using ion exchange chromatography and partially denaturing SDS−PAGE (Kanda et al., 1996). Some of these may be similar to those found in *A. bisporus* species because gill tissue contains isoforms found in cap skin, cap flesh, and stalk extracts (Figure 2).

Native Molecular Weight Determination. The native size of portabella tyrosinase was determined by size exclusion chromatography on Sephadex G-100. The native size was estimated to be ∼41 kDa when extrapolated from a plot of ln MW versus elution volume. This size compares favorably with that reported by van Gelder et al. (1997) and Wichers et al. (1996) but is smaller than the size estimated by Kanda et al. (1996) for *L. edodes* tyrosinase. The size of portabella tyrosinase in this study and that estimated by Wichers et al. (1996) is much different from the quaternary structure proposed by Strothkamp et al. (1976), in which the holoenzyme (H_2L_2, 120 kDa) contained two heavy chains (H, 43 kDa each) and two light chains (L, 13.4 kDa each).

Subunit Molecular Weight Estimation. The purified enzyme was subjected to SDS−PAGE under reduced and nonreduced conditions. The apparent subunit molecular weight was the same irrespective if the sample was reduced or not (Figure 3A) and was estimated to be ∼48 kDa. This is in general agreement with recent evaluations of the subunit molecular weight of tyrosinase (43 kDa) from *A. bisporus* (Wichers et al., 1995, 1996; van Gelder et al., 1997; Kumar and Flurkey, 1991), although the portabella tyrosinase may have a slightly larger molecular weight than *A. bisporus* tyrosinase. We did not find evidence of a light chain subunit as proposed by Strothkamp et al. (1976). A comparison of the subunit molecular weight obtained using the Laemmli system and an SDS−PAGE system run under more neutral conditions (the NUPAGE system, which was designed to prevent deamidation, alkylation, and oxidation during electrophoresis) showed sharper bands and the presence of a minor protein with slightly higher molecular weight in the NUPAGE system (Figure 3B). At present we do not know if this minor form with slighty larger molecular weight is another tyrosinase isoform. These results suggest that estimations of purity and subunit molecular weight may depend on the electrophoretic conditions or systems used. A similar finding was also reported for the tyrosinase in broad beans (Flurkey, 1990).

Western Blotting. Western blotting of the purified tyrosinase from portabella cap flesh revealed the enzyme to cross-react with antibodies against tyrosinase purified from *A. bisporus* U1 fruitbodies (Figure 3C). A major protein of ∼48 kDa was detected (Figure 3C, lane b). This immunological cross-reacting protein corresponded to the major protein in our purified preparation (lane c). A faintly stained immunological cross-reacting protein was also present in our purified sample and

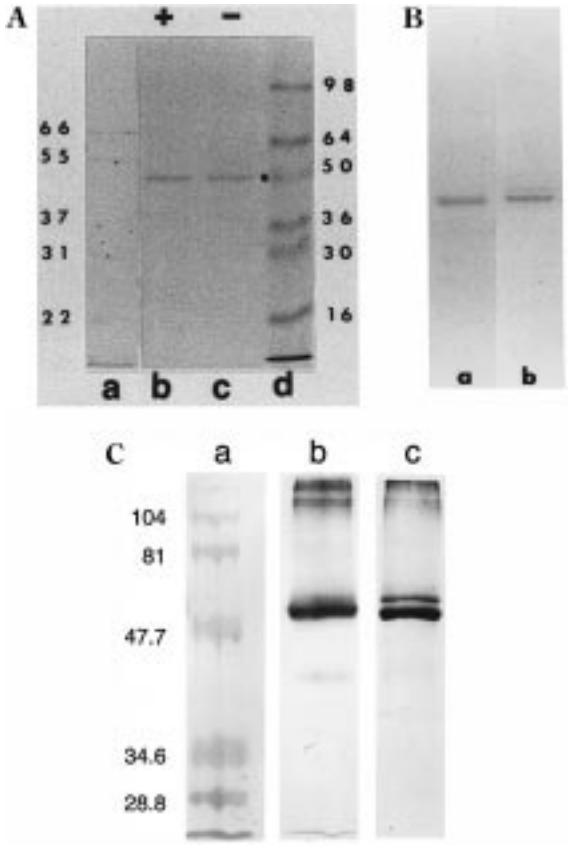

Figure 3. Denaturing electrophoresis and Western blotting of purified tyrosinase: (A) Tyrosinase was subjected to SDS−PAGE in 9% polyacrylamide gels in the presence (b, +) or absence (c, −) of 100 mM DTE using the Laemmli system. Lanes a and d contain protein MW standards. (B) Tyrosinase was subjected to SDS−PAGE in 9% polyacrylamide gels using the Laemmli system (a) and in 10% polyacrylamide gels using the NUPAGE system containing MOPS buffer (b) in the presence of 50 mM DTE. SDS−PAGE and NUPAGE were carried out as described under Materials and Methods. (C) Purified tyrosinase was subjected to Western blotting after SDS−PAGE as described under Materials and Methods. Lane a contained prestained molecular weight markers. Lane b contained purified portabella tyrosinase detected with antibodies against *A. bisporus* tyrosinase. Lane c contained the same purified portabella tyrosinase as in lane b but stained for protein.

showed a molecular size of ~40 kDa. A minor protein with similar molecular weight was also in the sample stained for proteins (lane c). The molecular weight of the tyrosinase from portabella flesh tissue as estimated from the Western blot corresponded, within limits or error, to the estimates from SDS−PAGE. This observation indicates structural similarities between tyrosinases from various *A. bisporus* strains and confirms earlier observations at the genetic level where tyrosinase sequences from *A. bisporus* U1 cross-hybridized with genomic sequences from other strains after Southern blot analysis (Wichers et al., 1995).

pH Optimum. Purified tyrosinase and tyrosinase in crude extracts showed maximum activity at pH 7.0. Activity dropped off sharply above pH 7.5, and no activity was present below pH 3.5. Half of the maximum activity was still present at pH 5.5 and 7.7. Similar findings were reported by Zhang and Flurkey (1999) for crude extracts of portabella tyrosinase. Cap skin extracts also showed a major optimum at pH 7.0, but a minor peak of activity was also observed at pH 3.5 (data not shown).

Table 1. Substrate Specificity of Cap Flesh Portabella Mushroom Tyrosinase[a]

substrate	concn (mM)	% activity relative to catechol
catechol	15	100
L-Dopa	15	57
D-Dopa	15	37
pyrogallol	15	3
catechin	5	5
chlorogenic acid	5	1
tyrosine	1	0.03

[a] Tyrosinase activity was measured spectrophotometrically as described under Materials and Methods. Values are expressed in percent activity relative to catechol in which 100% was equivalent to 0.115 unit/mL enzyme.

Substrate Specificity. The purified tyrosinase was able to use mono-, di-, and trihydroxy phenolic compounds as substrates. The enzyme showed low activity using mono- and triphenols as substrates but showed much greater activity with some types of diphenolic compounds (Table 1). In addition, the purified enzyme was also able to use the monophenol *p*-cresol as a substrate (data not shown). Because of its dual substrate and catalytic activities, the enzyme could be classified as a tyrosinase. The enzyme was most active with catechol as the substrate. L-Dopa showed higher activity than D-Dopa when used as substrate. The triphenol, pyrogallol, and diphenols, catechin and chlorogenic acid, showed much less activity compared to catechol. Very little activity was observed using tyrosine as a substrate.

Kinetic Studies. Both crude extracts and the purified enzyme displayed apparent Michaelis−Menten kinetics with catechol or L-Dopa as substrate. Nonlinear regression analysis of v versus [S] plots indicated that both the crude extract and purified enzyme were characterized with an apparent K_m for catechol of 5 mM. These values are slightly higher than the 2 mM found for the portabella cap skin tyrosinase (Zhang and Flurkey, 1999) and for the tyrosinase isozymes found in *L. edodes* (Kanda et al., 1996). Using the purified portabella tyrosinase, an apparent K_m for catechol of 5.5 mM was obtained using MBTH in conjunction with catechol as the substrate. In contrast, Espin et al. (1997) obtained a K_m for catechol of 0.44 mM using the MBTH assay system for the *A. bisporus* tyrosinase. The apparent K_m of purified portabella tyrosinase for L-Dopa was determined to be ~9 mM under assay conditions without MBTH. This is much higher than that reported for the tyrosinase in *A. bisporus* and *L. edodes*.

Inhibition Studies. A variety of inhibitors were examined to determine their potential for inhibition of tyrosinase activity. We have divided these inhibitors into the same groupings as reported by Ferrar and Walker (1996) (Table 2). Little to no inhibition was exhibited by 100 μM oxalic acid, pimelic acid, sorbic acid, γ-aminobutyric acid, anisidine, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, resorcinol, orcinol, EDTA, 2,2-dipyridyl, phenanthroline, *o*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, phenylacetic acid, phenylpyruvic acid, or caffeic acid. At 10 μM concentration, the most potent inhibitors were 2-mercaptobenzothiazole and salicylhydroxamic acid. Significant inhibition was observed using using either kojic acid, 2,3-dihydroxynaphthalene, 4-hexylresorcinol, tropolone, thiourea, cysteine, or L-mimosine at 100 μM concentration. These patterns of inhibition were very similar to those reported by Ferrar and Walker (1996) for inihibition of commercial grade mushroom tyrosinase.

Table 2. Inhibition of Cap Flesh Portabella Mushroom Tyrosinase[a]

	% original activity	
	100 μM inhibitor	10 μM inhibitor
none	100	100
aliphatic carboxylic acids		
ascorbic	0	88
kojic	13	57
amino compounds		
hydroxylamine	79	100
phenols		
2,3-dihydroxynaphthalene	20	68
4-hexylresorcinol	35	78
chelators		
tropolone	6	67
DIECA	28	71
sulfur compounds		
thiourea	12	62
cysteine	31	96
2-mercaptobenzothiazole	0	13
benzoic acids		
p-aminobenzoic acid	69	100
phenyl salicylate	83	100
miscellaneous		
SHAM		0
methimazole	83	100
L-mimosine	58	100
NADH	80	100

[a] Tyrosinase activity activity was measured spectrophotometrically as described under Materials and Methods using 15 mM catechol as the substrate. Percent activity is expressed relative to control assays without any inhibitor.

With the recent observations that commercial tyrosinase preparations contain other phenol oxidases (Sugumaran and Bolton, 1998; Flurkey et al., 1995; Kumar and Flurkey, 1991) and the finding of the existence of tyrosinase isoforms in different mushroom tissues (Zhang and Flurkey, 1997), there is a need for the characterization of purified tyrosinase isoforms. The data in this paper indicate that tyrosinase isolated from portabella cap flesh shares many similarities with the enzyme isolated from *A. bisporus*. These characteristics include its native molecular weight, subunit molecular weight, isoelectric point, immunological cross-reactivity, and inhibitor specificity. However, there are some differences between the two that include isoform composition, apparent substrate specificity, and apparent kinetic constants. The role or functions for these isoforms and their associated characteristics remain to be seen, but few tyrosinase isoforms have been purified to a single isoelectric p*I* isoform. Further work is needed to distinguish these subtle differences between isoforms purified from different tissues.

ABBREVIATIONS USED

DIECA, diethyldithiocarbamic acid; Dopa, 3,4-dihydroxyphenylalanine; DTE, dithiothreitol; IEF, isoelectric focusing; HA, hydroxylapaptite; MBTH, 3-methyl-2-benzothiazolinone hydrazone; MW, molecular weight; SHAM, salicylhydroxamic acid.

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