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Purification and characterization of tyrosinase from gill tissue of Portabella mushrooms

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Abstract

A group of tyrosinase isoforms with isoelectric points between 4.9 and 5.2 was isolated from gill tissue of Portabella mushrooms. Use of protease inhibitors was not able to increase the amount of latent forms significantly in crude extracts or to preserve latent tyrosinase activity during purification. The tyrosinase in gill tissue extracts showed latent activity above pH 5.5 and suppressed or displayed no latent activity below pH 5.5 when assayed in the presence of SDS. The purified isoforms showed monophenolase activity toward 4-hydroxyanisole but practically no activity toward tyrosine or tyramine. The purified isoforms showed greater activity toward catechol than either 4-methylcatechol, dopa, dopamine, chlorogenic acid, *t*-butylcatechol, or catechin. The *K*m for catechol was similar for the group of isolated isoforms (4.3 mM) compared to the isoforms in crude extracts (5.3 mM). Crude extracts showed several isoforms ranging from 50 to 230 kDa after partially denaturing SDS PAGE, while the purified isoforms showed molecular weights of 70 kDa.

Keywords: *Agaricus bisporus*; Portabella mushrooms; Gill tissue; Tyrosinase; Purification

1. Introduction

The enzyme tyrosinase (E.C. 1.14.18.1) is a member of the tyrosinase related protein family and is found throughout the phylogenetic scale of living organisms (for a review of tyrosinase and tyrosinase related proteins, see del Marmol and Beermann, 1996; Garcia-Borron and Solano, 2002; Hearing and Tsukamoto, 1991; van Gelder et al., 1997). Tyrosinase performs a variety of functions in these organisms ranging from pigmentation to defense to sclerotization. The enzyme catalyzes at least two different reactions using phenolic compounds and molecular oxygen as substrates. These reactions include hydroxylation of monophenols to form *ortho*-diphenols (monophenolase or cresolase activity) and oxidation of diphenols to form *ortho*-diquinones (diphenolase or catechol oxidase activity). It has also been suggested that tyrosinase can oxidize 5,6-dihydroxyindole to the 5,6-dihydroxyquinone (Korner and Pawelek, 1982).

There are several oxidases that can use phenolic substrates present in mushrooms, but tyrosinase is probably the principal enzyme involved in browning reactions in *Agaricus bisporus* (review by Jolivet et al., 1998). For example, tyrosinase, laccase, and peroxidase were detected in Portabella mushrooms, a brown strain of *Agaricus bisporus*, but tyrosinase was present in larger amounts than either laccase or peroxidase (Zhang and Flurkey, 1997). These authors also found that more tyrosinase was present in the stalk than in the cap skin, gill tissue, or cap flesh tissue. However, it is difficult to accurately determine quantitative tyrosinase activities associated with different tissues because of latent and active enzyme forms, the difficulty of determining protein content, and different isoform composition at different developmental stages (Jolivet et al., 1998; Van Leeuwen and Wichers, 1999). Zhang and Flurkey (1999) also showed that crude extracts of different mushroom tissues showed a slightly different isoform composition after analysis by isoelectric focusing (IEF). Crude gill tissue extracts showed less of the low pI isoforms and more of the higher pI isoforms (Zhang and Flurkey, 1999; Zhang et al., 1999). Tyrosinase isolated from cap skin and cap flesh of Portabella mushrooms and from

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Agaricus bisporus has a subunit molecular weight of around 43–48 kDa (Zhang and Flurkey, 1999; Ylostalo et al., 2001; Gerritsen et al., 1994; Wichers et al., 1996), although latent enzyme can be isolated with a higher molecular weight using freshly picked/frozen *Agaricus bisporus* tissue (Espin and Wichers, 1999).

Gill tissues contain abundant melanized spores in mature mushrooms. γ-glutaminyl-4-hydroxybenzene (GHB) is considered to be the main substrate for melanogenesis catalyzed by tyrosinase/phenolase in *Agaricus bisporus* (Rast, et al., 1981). Tyrosinase has been shown to hydroxylate GHB into γ-glutaminyl-3,4-dihydroxybenzene (GDHB) and then to oxidize GDHB into γ-glutaminyl-3,4-benzoquinone during the melanization process of sporogenesis (Boekelheide et al. 1979, 1980; Rast et al., 1981). However, the fate and physiological role of GHB may be different in mycelium (Soulier et al., 1993; Jolivet et al., 1998). Tyrosinase has been located in "tyrosinase vesicles" associated with cell walls and perhaps in cell wall regions containing amorphous melanin as well (Rast et al., 2003). Since melanin deposition in fungi takes place in extracellular locations, tyrosinase may play a key role in the development of the fungal cell wall constituents. Perhaps pigmentation in the different extracellular cell wall locations and in spores relies on different tyrosinase isoforms or isoforms with different substrate specificities. Characterization of the enzyme located in the tissues, i.e. gills, involved in converting GHB into GDHB and further oxidation into melanin may provide further insight into the synthesis of spore melanins and melanization. This study is one of the first to examine some of the biochemical characteristics associated with tyrosinase isolated from gill tissue of Portabella mushrooms.

2. Results and discussion

Purification of tyrosinase from gill tissue is somewhat more difficult due to the smaller amount of tissue available per mushroom and greater amount of pigmentation in these tissues. Initially, we tried various adsorbents (polyvinylpyrrolidone, Polyclar AT, XAD nonionic adsorbents, AG anion exchangers, and celite) as well as precipitation methods (polyethyleneglycol, acetone, ammonium sulfate, and ethanol) to remove colored material or to concentrate tyrosinase. None of these methods were successful without causing a decrease in the recovery of enzyme activity or not being able to remove a substantial amount of colored material. Secondly, in order to isolate a group of closely related pI tyrosinase isoforms, selective pooling from column chromatography was also necessary. As a result, lower recovery and yields were obtained. Lastly, both latent and active enzymes were present in crude extracts. This is rather surprising to find latent enzyme in these tissues given that store-bought mushrooms usually have little latent enzyme in them. However, even with the use of protease inhibitors, we were not able to prevent activation of latent enzyme during purification.

Table 1 shows the effect of using protease inhibitors on the extraction of latent tyrosinase from gill tissues. Approximately half of the enzyme was latent in extracts prepared in the absence of protease inhibitors. Preparation of extracts in the presence of protease inhibitors that would inhibit serine proteases, metallo-proteases, and cysteine proteases using PMSF, EDTA, and E-64, respectively, did not increase the percentage of latent enzyme appreciably although the total activity increased. Use of pepstatin to inhibit acidic proteases had no appreciable effect on increasing latent activity. These results indicate that at least half of the enzyme had already been converted into an active form in the tissue samples irrespective of what type of inhibitor was used. During the purification of tyrosinase, we were not able to preserve latent enzyme, and most of the latent enzyme was converted into active enzyme (data not shown). We also noticed that the amount of latent enzyme present in the isolated fractions varied from preparation to preparation. Presumably this variation is a result of limited proteolysis or partial unfolding of the latent enzyme according to the model of the maturation and activation of latent *Agaricus bisporus* mushroom tyrosinase proposed by Espin and Wichers (1999) and Espin et al. (2000a). In contrast, Espin et al. (1999) were able to isolate a latent tyrosinase from freshly harvested and frozen *Agaricus bisporus* mushrooms without the use of protease inhibitors. This latent enzyme could be activated in vitro by trypsin, a serine protease. To date

Table 1
Effect of protease inhibitors during the extraction of gill tissue tyrosinase

Protease inhibitor	SDS	Total tyrosinase activity (units/ml)	Latent tyrosinase activity (units/ml)	% Latent activity
Control	+	15.7	8.6	55
	−	7.1		
PMSF (1 mM)	+	22.7	13.9	61
	−	8.8		
EDTA (1 mM)	+	20.8	12.9	62
	−	7.9		
E-64 (1 μg/ml)	+	20.0	12.3	62
	−	7.7		
pepstatin (1 μg/ml)	+	17.8	9.9	56
	−	7.9		

Crude extracts, with and without the presence of added inhibitors, were prepared by blending 10 g of tissues with 100 ml of 10 mM phosphate buffer (pH 6.5), centrifuging the homogenate, and assaying the supernatant for active and latent tyrosinase activity as described in the material and methods. Latent activity is the activity detected in the presence of 0.1% SDS minus the activity detected in the absence of 0.1% SDS.

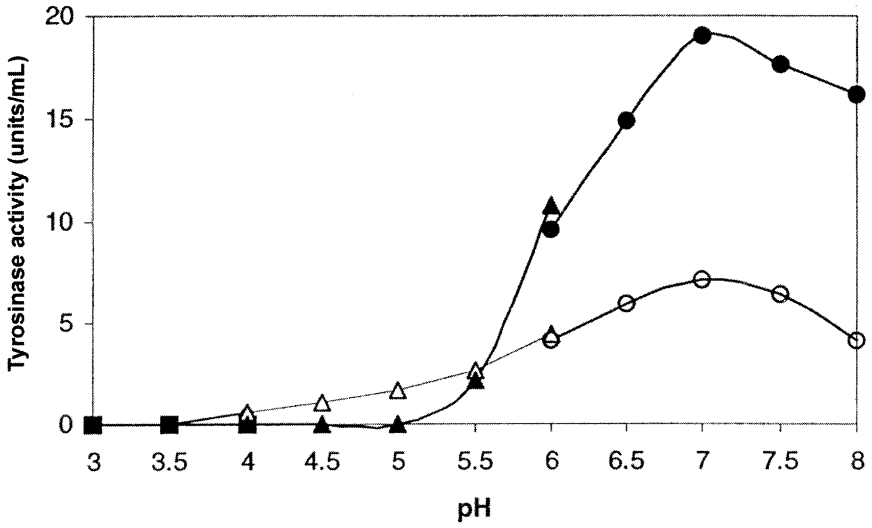

Fig. 1. Effect of pH and SDS on gill tissue tyrosinase activity. Tyrosinase activity was measured using 10 mM catechol as the substrate, with or without 0.1% SDS, at various pH as described in the experimental section. Open symbols refer to assays without SDS. Filled symbols refer to assays with SDS. Square symbols refer to citrate buffers. Triangle symbols refer to acetate buffers, and circles refer to phosphate buffers.

this is the only group of investigators able to isolate a fully latent tyrosinase from mushrooms.

The latent tyrosinase activity measured in crude extracts from gill tissues is dependent upon the pH of the assay medium. At pH values below 5 there was little to no latent activity detected when assayed in the presence of SDS, although there was some activity detected in the absence of SDS (Fig. 1). Above pH 6.0 the amount of tyrosinase activity was significantly greater than that assayed in the absence of SDS, indicating a greater amount of detectable latent enzyme. At more acidic pHs the ability to be activated by SDS was suppressed. The pH optimum was approximately pH 7.0 when assayed in the presence and absence of SDS for total and active tyrosinase activity, respectively. Latent *Agaricus bisporus* tyrosinase isolated by Espin et al. (1999) did show a small peak of activity from pH 4–4.5 in the absence of SDS due to acid shocking. However, the latent activity in gill tyrosinase (this report) showed no such peak of activity at low pH in the absence of SDS. It is possible that the brown strain of mushrooms may show different activation/latency characteristics of tyrosinase at lower pH compared to the tyrosinase found in the white strain of *A. bisporus*. SDS activation profiles of latent tyrosinase/polyphenoloxidase versus pH were also reported in broad beans (Moore and Flurkey, 1990; Jimenez and Garcia-Carmona, 1996), apples (Marques et al., 1995), *Prunus* fruits (Fragnier et al., 1995), and persimmon (Nunez-Delicado et al., 2003). In general, they showed similar activation by SDS at higher pH, similar suppression of activation at lower pH, and, in some cases, a pH optimum at acidic pH values when assayed in the absence of SDS. This pH

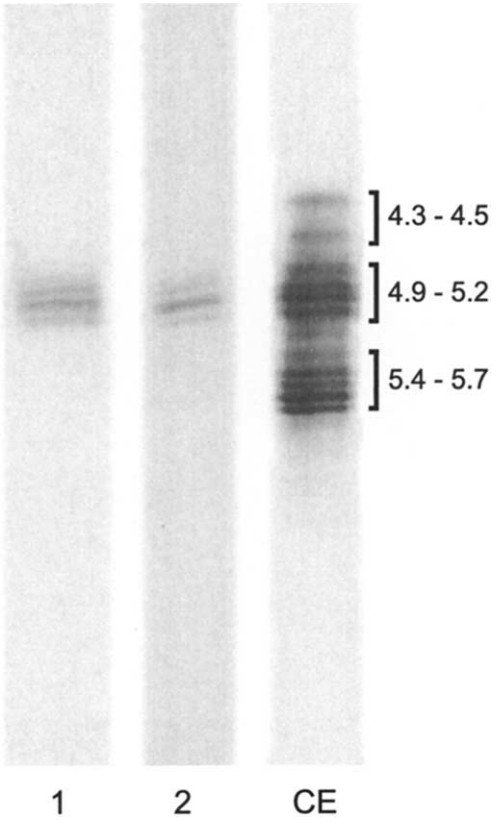

Fig. 2. Isoelectric focusing of crude and purified gill tissue tyrosinase. Isoeletric focusing was carried out in pH 3–7 gradients at 10 °C. Gels were stained with 1 mM catechol and 2 mM MBTH. pI values were determined by comparison to pI standards. 1 and 2 refer to two different HA purified preparations of gill tissue tyrosinase. CE refers to a crude extract of gill tissues. pI groupings are designated by brackets.

dependency on latency, SDS, and acid shocking may represent different mechanisms for activation.

Crude gill extracts contained several pI isoforms that clustered around pH 4.9–5.2 and another group of isoforms that clustered around pH 5.4–5.7 (Fig. 2). Only small amounts of the lower pI isoforms around pH 4.3–4.5 were present in gill tissues. There were approximately four heavily stained isoforms present in the 4.9–5.2 pI range and four heavily stained isoforms in the 5.4-5.7 pI range. At least 16 different isoforms with varying intensity could be identified by IEF in crude extracts after prolonged staining. This number is larger than that reported by van Leeuwen and Wichers (1999) for tyrosinase in crude extracts of gill tissues from *Agaricus bisporus* and larger than that in an earlier report by Zhang and Flurkey (1999) using crude extracts of gill tissues from Portabella mushrooms. Two different tyrosinase preparations that were isolated following the purification procedure outlined above contained the same 3–4 isoforms that clustered between pI 4.9–5.2. Although we only examined active IEF isoforms from these preparations, the distribution of individual IEF forms within the 4.9–5.2 pI cluster was slightly different. This suggests that there may be some conversion or loss of these isoforms during the purification process that we could not prevent and illustrates the difficulty in isolating specific tyrosinase isoforms.

Chromatography on DEAE columns can partially separate many of the isoforms with higher and lower pIs. Using DEAE columns equilibrated at pH 7, tyrosinase was eluted in the order of high to low pI isoforms with increasing salt concentrations. Further separation of the isoforms was achieved using hydroxyapatite (HA) chromatography (Fig. 3). Depending on what fractions were pooled from the DEAE column,

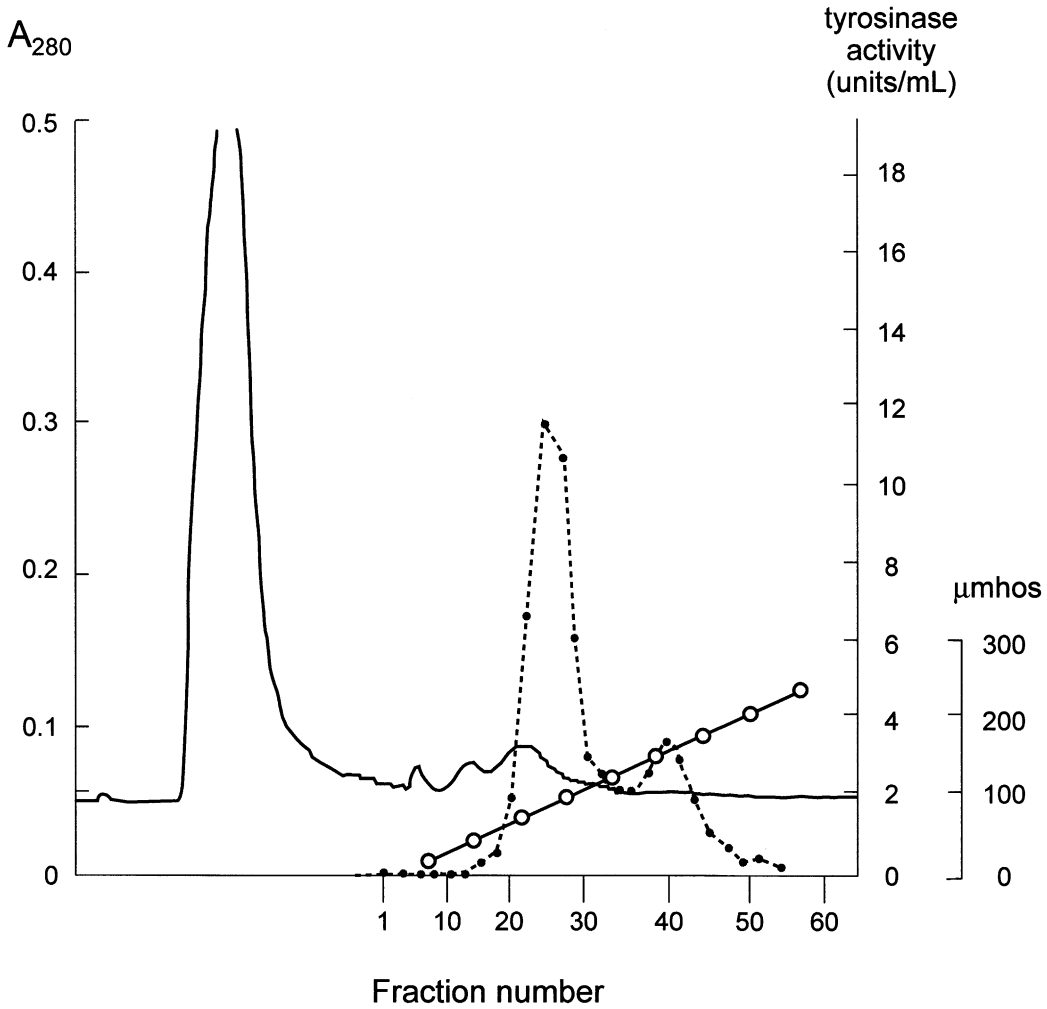

Fig. 3. Chromatography of gill tyrosinase on hydroxyapatite. Selective fractions from tyrosinase subjected to chromatography on DEAE cellulose were applied to a hydroxyapatite column equilibrated in 10 mM phosphate buffer (pH 7.0). Tyrosinase activity was eluted with a 10–250 mM phosphate buffer gradient. Fractions containing the group of IEF isoforms from 4.9 to 5.2 (first peak) were pooled and used for further experiments. Solid line denotes absorbance at 280 nm. Dashed line represents tyrosinase activity. Open circles designate conductivity of phosphate buffer gradient in diluted samples from fractions.

HA chromatography resulted in two to three peaks of tyrosinase activity with middle to higher pI forms eluting later in the column fractions. Fig. 3 shows two major peaks of tyrosinase activity and a very minor peak at fraction 51 eluting during HA chromatography. The order of elution of tyrosinase isoforms using HA chromatography was from low to middle to high pI isoforms with increasing phosphate buffer concentrations. Isoelectric focusing of two different purification attempts showed that the middle group of isoforms, pI 4.9–5.2, was separated from lower and high pI isoforms (Fig. 2). However, there were still several isoforms (3–4) present with minor differences in pI. Although several isoforms were detected by IEF in the purified samples, these same samples showed a single, albeit smeary, band of tyrosinase activity after native PAGE (data not shown). This indicates that the isoforms have similar sizes and charges. At present, we have not been able to separate the members of this isoform group from one another using conventional chromatographic methods.

In spite of the isoform separation, the material obtained from HA chromatography was not completely homogenous when analyzed by SDS PAGE (Fig. 4 a). The purification scheme removed most of the major proteins with lower molecular weight found in the crude extracts (Fig. 4a HA vs CE). Two major stained bands of protein were present in the final sample along with a protein with less staining intensity. The estimated sizes of the major bands were 48 and 43 kDa, respectively. The minor protein band was estimated to have a size of 60 kDa. Several minor proteins smaller than 45 kDa were present but not readily visible in the scanned gels. We assume that the bands in the 43–48 kDa are the active proteolyzed forms of tyrosinase that many other investigators have observed (Wichers et al., 1996; Gerritsen et al., 1994; Zhang et al., 1999). The minor band at 60 kDa could be the latent tyrosinase that is known to have a higher molecular weight (Espin and Wichers, 1999; Espin et al., 2000a). Alternatively, this 60 kDa protein could be a protein contaminant still remaining in the preparation. Partial protein sequencing or Western blotting with specific antibodies would be needed to confirm this potential relationship. Recently, we have been able to remove some 50–70 kDa proteins from commercial tyrosinase preparations using hydrophobic interaction chromatography (data not shown). Since the commercial tyrosinase contains little latent enzyme, this suggests that the 60 kDa protein in the isolated gill tyrosinase could be a contaminant rather than a latent enzyme form.

Under partially denaturing SDS PAGE, samples not boiled or treated with reducing agents, several isoforms of tyrosinase were detected in crude extracts of gill tissue (Fig. 4b). The size of these isoforms ranged from 50 to 230 kDa when stained for enzyme activity. A broad band at approximately 70 kDa showed heavy staining.

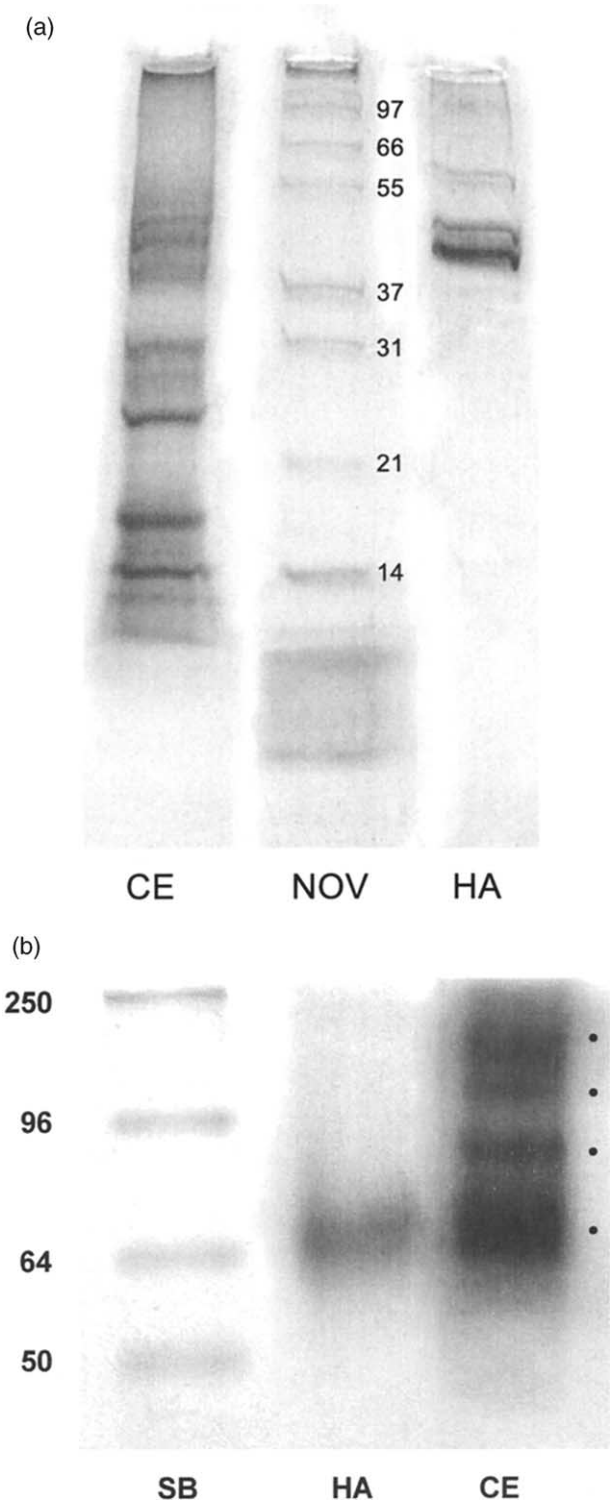

Fig. 4. (a) Denaturing SDS PAGE of crude and purified tyrosinase from gill tissue. Samples from the crude extract (CE) and tyrosinase purified through hydroxyapatite chromatography (HA) were subjected to SDS PAGE using the Laemmli method. Protein standards (NOV) were used to estimate molecular weights. (b) Partially denaturing SDS PAGE of crude and HA purified tyrosinase from gill tissue. Samples from CE and HA, not denatured, were subjected to SDS PAGE and stained for tyrosinase activity with 2 mM dopa/2 mM MBTH in the presence of 0.1% SDS. See-Blue (SB) pre-stained molecular weight protein standards were from Invitrogen (formerly Novex).

The purified gill tyrosinase isoforms also showed a single band at this same position, suggesting that the pI 4.9–5.2 IEF isoforms have an apparent MW of 70 kDa under these conditions. The other isoforms observed after partially denaturing SDS PAGE were apparently removed during the purification procedure. Native PAGE showed three broad bands of staining activity in crude extracts and one broad band in the purified gill tyrosinase fractions that corresponded to the major stained band in crude extracts (data not shown). Even though the subunit size determined by denaturing SDS PAGE of purified gill tyrosinase was 43–48 kDa, partially denaturing SDS PAGE indicated a much larger size. This increase in size could be due to conformational changes and/or aggregation of monomeric units. However, conformational changes would not likely account for those isoforms with apparent sizes larger than 70 kDa, especially if the subunit molecular weight is 43–48 kDa. The results from partially denaturing SDS PAGE, SDS PAGE, IEF, and native PAGE illustrate the difficulty in trying to determine tyrosinase isoform composition and characteristics of specific tyrosinase isoforms.

Tyrosinase activity in crude extracts and in the partially purified sample was examined with regard to its monophenolase and diphenol oxidase activity Table 2. Both the crude extracts and purified enzyme were able to hydroxylate monophenols and oxidize diphenols. 4-Hydroxyanisole was the substrate that showed the most activity based on a relative comparison of the three monophenols tested for monophenolase activity. However, the monophenolase activity was very low in the purified samples, indicating a substantial loss of this activity during the purification. We have observed that this activity was lowered further when assayed in the presence of SDS, suggesting that the monophenolase activity is more susceptible to conformational changes around the active site than the catechol oxidase activity (data not shown). The labile nature of the monophenolase activity of tyrosinase has been noted in many earlier reports.

Table 2
Monophenolase and diphenol oxidase activity of crude and partially purified gill tyrosinase

	Relative activity (%)	
	Crude enzyme	Partially purified enzyme
Monophenols		
4-hydroxyanisole	100 (16.1)	100 (0.28)
tyrosine	<1	<1
tyramine	3	<1
Diphenols		
catechol	100 (5.6)	100 (1.9)
4-methylcatechol	59	22
DL-dopa	23	21
dopamine	31	35
chlorogenic acid	39	36
t-butylcatechol	13	15
catechin	12	11

Tyrosinase activity was measured spectrophotometrically as described in the experimental section. Relative activities were based on the substrate with the greatest amount of activity as 100%. Numbers in parentheses refer to actual enzyme activity in units/ml.

Of the diphenols tested, highest activity was shown with catechol as the substrate using either crude extracts or purified enzyme. Except for 4-methyl catechol, a similar percent relative activity was observed with dopa, dopamine, chlorogenic acid, *t*-butylcatechol, or catechin as substrates using crude extracts compared to the purified enzyme. This indicates that purification did not change the general substrate preferences of tyrosinase. The overall relative diphenol oxidase activity decreased approximately 67% when the enzyme was purified compared to the crude extract. This decrease in diphenol oxidase activity (5.6 units/ml vs 1.9 units/ml) was not nearly as great as the decrease observed in the monophenolase activity (16.1 units/ml vs 0.28 units/ml). In addition, much of the latent tyrosinase activity had disappeared in the purified preparations compared to crude extracts (data not shown). In general, these results are in agreement with those found for the tyrosinase IEF isoforms (pI 5.1–5.3) isolated from cap flesh of Portabella mushroom (Zhang et al., 1999) but not as similar for the tyrosinase IEF isoform (pI 4.3) isolated from cap skins of Portabella mushrooms (Ylostalo et al., 2001).

Using catechol as the substrate, the *K*m was determined for active tyrosinase from crude extracts and from the purified tyrosinase preparations. In the absence of SDS in the assays, the tyrosinase in crude extracts and in purified preparations showed apparent hyperbolic curves in velocity versus substrate plots over a broad range of catechol concentrations (Fig. 5). In the absence of SDS, the apparent *K*m of catechol for tyrosinase in crude extracts was determined to be 5.3 mM while a *K*m of 4.3 mM for catechol was obtained using tyrosinase in purified samples. The *K*m for crude tyrosinase extracts assayed in the presence of SDS to detect total tyrosinase activity was 3.6 mM (data not shown). These *K*m values determined in the absence of SDS represent contributions from all active nonlatent tyrosinase forms in each sample. The *K*m values determined in the presence of SDS represent contributions from all active and activated forms of tyrosinase. Based on the above *K*m values, there may be a relationship between *K*m and the forms of tyrosinase that are activated, but this requires further study to confirm this possibility. The Km values for Portabella gill tyrosinase in this study seem to be somewhat greater than those reported for tyrosinase from *A. bisporus* (Espin and Wichers, 1999; Espin et al., 2000a). Even though sub-

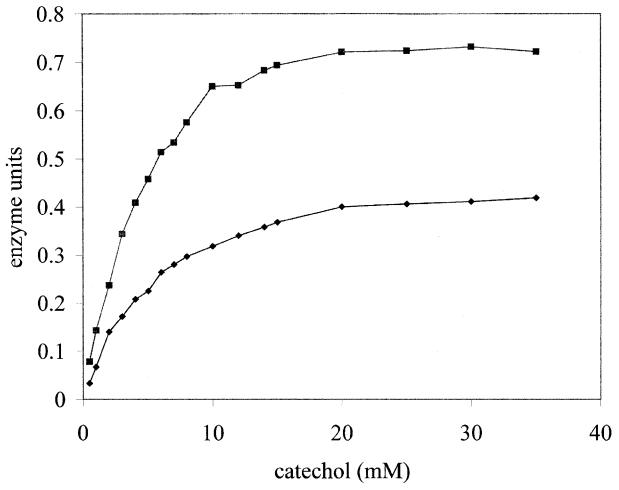

Fig. 5. Velocity vs [catechol] plot of crude (dark colored squares) and purified (light colored squares) gill tissue tyrosinase. Tyrosinase activity of the crude gill tissue extracts was measured in the presence of different catechol concentrations ranging from 0.5 to 50 mM. Tyrosinase activity of the purified tyrosinase was measured in the presence of catechol concentrations ranging from 0.5 to 35 mM. No SDS was included in the assays. *K*m and *V*max values were determined from nonlinear regression analysis using Enzpack software.

unit molecular weights and IEF isoform composition of tyrosinase seem to be similar in the brown strain of *A. bisporus,* Portabella mushrooms, compared to the white strain of *A. bisporus*, there may be some underlying differences in their kinetic properties and substrates preferences.

3. Experimental

3.1. Materials

Fully expanded large caps (ca. 10 cm diameter) from Portabella mushrooms were obtained from local groceries. The gill tissue was scraped from the caps, frozen in liquid nitrogen, and stored at −80 °C until processed. Protease inhibitors were obtained from Sigma Chemical Co., St. Louis, MO. Isoelectric focusing gels were obtained from Hypure Inc., Norton, OH, and from BioWhittaker Molecular Applications, Rockland, MA. Hydroxyapatite HTP was purchased from BioRad Laboratories, Hercules, CA.

3.2. Purification of tyrosinase from gill tissue

Gill tissue tyrosinase was purified using modifications of the methods for isolating tyrosinase from the cap skin and cap flesh tissue of Portabella mushrooms as reported by Ylostalo et al. (2001) and Zhang and Flurkey (1999). Briefly, crude extracts were prepared by grinding 50–100 g of tissue into a powder using liquid nitrogen. The powder was homogenized in five volumes of 10 mM sodium phosphate buffer (pH 6.5) containing the following protease inhibitors: 1 mM PMSF, 1 mM EDTA, 1 μg/ml of pepstatin A, and 1 ug/ml of E-64. After centrifugation, the supernatant was applied onto a DEAE cellulose column (2.5 × 12 cm) equilibrated in 10 mM phosphate buffer (pH 7.0). Enzyme was eluted with a 0–500 mM NaCl gradient. After SDS PAGE and IEF analysis of column fractions, selective pooling of various tyrosinase IEF group isoforms was carried out. The tyrosinase group of IEF isoforms in the range of pI 4.9–5.2 was concentrated and applied onto a hydroxyapatite column (1 × 9 cm) equilibrated in 10 mM sodium phosphate buffer (7.0). Tyrosinase isoforms were eluted with a 10–250 mM phosphate buffer gradient. After IEF and SDS PAGE analysis, isoforms in the pI range of 4.9–5.2 were collected for further characterization. In some cases, it was necessary to repeat the hydroxyapatite chromatography to remove other tyrosinase IEF isoforms present in minor amounts.

3.3. Enzyme assays

Tyrosinase activity was monitored spectrophotometrically using catechol as the substrate as reported earlier (Zhang et al., 1999). One unit of enzyme activity was defined as a change in one absorbance per min at 410 nm. Stock solutions of catechol were prepared in 0.5 mM H_3PO_4. Assays were conducted in the presence and absence of 0.1% SDS to detect latent enzyme activity. Latent activity is the amount of enzyme detected in the presence of SDS minus the amount detected in the absence of SDS. Monophenolase activity was determined using tyrosine (484 nm), tyramine (476 nm), and 4-hydroxyanisole (464 nm) as substrates along with MBTH in 50 mM phosphate buffer (pH 6.5) as described by Espin et al. (2000b) and Rodriquez-Lopez et al. (1994). The substrate concentrations were 0.5 mM and the MBTH concentration was 2 mM. Diphenol oxidase activity was determined using 4-methylcatechol (410 nm), catechol (410 nm), DL-dopa (475 nm), dopamine (480 nm), chlorogenic acid (400 nm), *t*-butylcatechol (400 nm), and catechin (390 nm) as substrates in 50 mM sodium phosphate buffer (pH 6.5) as described by Zhang et al. (1999). The final substrate concentrations were 5 mM. One unit of enzyme was defined as a change of one absorbance unit per min.

3.4. Kinetic constants

Kinetic constants were determined using catechol as the substrate. Catechol concentrations ranged from 0 to 55 mM and rates were determined from the linear portion of the absorbance versus time curves. Kinetic constants were evaluated using non-linear regression analysis by the method of Wilkinson in the Enzpack

software (Biosoft, Cambridge, UK). Kinetic constants were determined using crude extracts and the purified enzyme in the presence of absence of 0.1% SDS in the assay mixtures.

3.5. Electrophoresis and isoelectric focusing

Native PAGE was carried out using the Laemmli SDS PAGE system without SDS (Chen and Flurkey, 2002) and under partially denaturing conditions (with SDS, no reducing agent, samples not boiled). SDS PAGE was carried out using the Laemmli system in which samples were denatured and treated with 50 mM dithioerythreitol (DTE, final concentration) before application (Zhang et al., 1999). Isoelectric focusing was carried out as described earlier by (Zhang et al., 1999) and more recently by Chen and Flurkey (2002). IEF gels were stained for tyrosinase activity using 1 mM catechol and 2 mM MBTH and destained in water.

3.6. pH optimum and pH effect on SDS activation

The pH optimum of tyrosinase was determined using catechol as in substrate. All buffer concentrations were 100 mM. Sodium citrate buffers were used to encompass a pH range from 3 to 4. Sodium acetate buffers were used for pH ranges from 4 to 6, and sodium phosphate buffers were used in pH ranges from 6 to 8. All assays were conducted in the presence and absence of 0.1% SDS.

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