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# Enzyme, Protein, Carbohydrate, and Phenolic Contaminants in Commercial Tyrosinase Preparations: Potential Problems Affecting Tyrosinase Activity and Inhibition Studies

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Commercial mushroom tyrosinase contains other proteins, enzymes, carbohydrates, and phenolic material besides tyrosinase. Carbohydrate and phenolic material comprise a large percentage of the powder resuspensions derived from *Agaricus bisporus*. Enzyme assays identified the presence of tyrosinase, laccase, $\beta$-glucosidase, $\beta$-galactosidase, $\beta$-xylosidase, cellulase, chitinase, xylanase, and mannanase in the commercial tyrosinase. Protein sequencing indicated the presence of tyrosinase, a lectin, and a putative mannanase as well as 10 unidentified protein/peptides in the commercial tyrosinase preparations. Characteristics of tyrosinase isoforms were similar in two different commercial tyrosinase sources. Inhibition studies indicated that $I_{50}$ values for some tyrosinase inhibitors were different when the crude powder was compared to a partially purified tyrosinase. The presence of these contaminants has the potential to affect studies using commercial tyrosinase.

**KEYWORDS:** ***Agaricus bisporus*; tyrosinase; phenolics; carbohydrates; proteins; glycosidases; inhibitors**

## INTRODUCTION

Tyrosinases are found in bacteria, fungi, plants, vertebrates and invertebrates, and mammals, although sometimes it has been difficult to differentiate them from polyphenol oxidases or catechol oxidases [for reviews see Claus and Decker (*1*), Halaouli et al. (*2*), van Gelder et al. (*3*), Marusek et al. (*4*), Yoruk and Marshall (*5*), and Sugumaran (*6*)]. Tyrosinase (monophenol; *o*-diphenol:oxygen oxido-reductase, EC 1.14.18.1) is a copper-containing enzyme that catalyzes the first step in melanin biosynthesis. Tyrosinase and tyrosinase-related proteins are intimately linked with pigmentation in mammals [reviewed in del Marmol and Beerman (*7*)]. In plants, tyrosinases and/or polyphenol oxidases have been suggested to participate in wound healing, defense reactions [reviewed in van Gelder et al. (*3*), Marusek et al. (*4*), and Yoruk and Marshall (*5*)] and, more recently, in the synthesis of flower pigments (*8*). In fungi, tyrosinases have been postulated to participate in spore formation, defense reactions, and pigmentation [reviewed in Halaouli et al. (*2*)].

Because of the reactions and functional roles associated with tyrosinase, the enzyme has been used as a tool or as a model system in many studies [reviewed in Halaouli et al. (*2*), Kim and Uyama (*9*), Rescigno et al. (*10*), and Seo et al. (*11*)]. Our examination of recent studies (2003−present) using mushroom tyrosinase (MT) found that approximately 80% of these studies used crude commercial tyrosinase preparations derived from the common button mushroom *Agaricus bisporus*. Only a fraction of these studies attempted to use a purified or partially purified form of the enzyme. In some of these studies, the enzyme was used to detect phenol in wastewater and generate phenols for other applications (*11*), to quantitate acetaminophen (*12*), to examine tyrosinase structure−activity relationships in combinatorial libraries (*13*), to assess structural stability of tyrosinase in the presence of SDS (*14*), to demonstrate that egg white lysozyme is an inhibitor of mushroom tyrosinase (*15*), and in a Raman spectroscopy study of the cresolase activity of tyrosinase (*16*). Many other papers have used commercial tyrosinase for examining inhibitory characteristics of compounds isolated from natural products or chemically synthesized compounds (*9*–*11*). Marrero-Ponce et al. (*17*) have a fairly comprehensive list of tyrosinase inhibitors in their study using atom-based bilinear indices as predictors for inhibiting tyrosinase activity. In general, there seems to be an increasing use of the commercial tyrosinase to screen naturally occurring products and synthetic inhibitors for use as skin-whitening/antibrowning agents.

Earlier studies have shown that crude extracts of *A. bisporus* contain other phenol-oxidizing enzymes, including laccase and peroxidase (*18*–*20*). Because these enzymes can be found in crude extracts of *A. bisporus*, they may be present in commercial

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tyrosinase preparations. Contamination of commercial tyrosinase by laccase is especially important because laccase can use some of the same substrates as tyrosinase (e.g., dopa) and may interfere with determination of tyrosinase activity. Laccase contamination varied in different lots of MT from the same supplier and was responsible for a quinone methide formed from 2,6-dimethoxy-4-allylphenol that was formerly attributed to tyrosinase (*21*). More recently, laccase has been found in several commercial MT preparations, confirming these earlier findings (*22*). Contamination by any enzyme that can affect estimations of tyrosinase activity will in turn cast doubt on any kinetic or inhibition data gathered using mushroom tyrosinase, commercial or otherwise.

Commercial MT preparations may contain a variety of other enzymes that could act upon tyrosinase directly (e.g., proteases) or act upon tyrosinase substrates and/or inhibitors (e.g., phenol oxidases, glycosidases). Proteases are known to convert latent *A. bisporus* tyrosinase into an activated form with a smaller molecular weight (*3, 4, 23*). Use of protease inhibitors can prevent this conversion and also change tyrosinase isoform composition and distribution in the enzyme extracted from *A. bisporus* strains (*23, 24*). These results suggest the commercial tyrosinase lacks most of the latent enzyme and has already been converted by proteolysis into an activated form with a smaller molecular weight.

A contaminating glycosidase in the commercial MT preparation could convert a glycosylated phenolic compound into aglycone that could potentially interfere with tyrosinase inhibitor studies. For example, licuraside and isoliquiritin, both glycosylated phenols, have been reported to be inhibitors of mushroom tyrosinase (*25*). Hydrolysis of these inhibitors by a contaminating glycosidase could possibly convert a tyrosinase inhibitor into a non-tyrosinase inhibitor and affect interpretations of $K_i$ and $I_{50}$ values. It is also possible for a noninhibitory phenolic glycoside to be converted into an inhibitory phenolic compound by a glycosidase. At least one example of this possibility has been reported. The phenolic compound escutelin is a competitive inhibitor of mushroom tyrosinase with a $I_{50}$ of 43 $\mu$M, whereas the glycoside derivative, esculin, shows relatively little inhibition toward mushroom tyrosinase with an $I_{50}$ value of >14 mM (*26*). Recently, Rescigno et al. (*22*) showed that a mixture of esculin and a $\beta$-glucosidase could affect catalytic rates of MT using dopa as a substrate.

Other contaminants in commercial MT preparations may also affect tyrosinase activity. Organic compounds, including phenols, polyphenolic material, and carbohydrates can be chemically reactive. They can potentially modify tyrosinase and its substrates or inhibitors through a variety of chemical reactions. This is especially of concern to the food industry with regard to food browning and its prevention (*27*). Some of these polymers may also act as adsorbents that could bind tyrosinase or its substrates/inhibitors. Most reports using commercial MT have not alluded to potential problems associated with the possible presence of these nonprotein/enzyme compounds.

Lastly, *A. bisporus* tyrosinase extracted from live mushrooms (*28*) and commercial tyrosinase preparations (*19*) has been shown to contain a variety of isoforms. These isoforms may or may not have similar kinetic and inhibition characteristics with regard to tyrosinase activity. Investigators using commercial sources of tyrosinase rarely comment on this problem.

If one uses commercial MT for various studies, it is important to know what one is dealing with. Our goal in this study was to examine two different sources of commercial MT with regard to enzyme/protein contamination, protein content, carbohydrate content, phenolic content, and tyrosinase characteristics. Few investigators may have thought about how these contaminants could affect their results and interpretations of those results.

## MATERIALS AND METHODS

**Materials.** Commercial mushroom tyrosinase preparations were obtained from Sigma Chemical Co. (SMT, lot 105K7026; St. Louis, MO) and Worthington Biochemical Corp. (WMT, lots 33H6588Q and 36E8802; Lakewood, NJ). Precast SDS−polyacrylamide gels and isoelectric focusing gels were obtained from Cambrex Bio Science Rockland, Inc. (Rockland, ME). Coomassie protein assay kits were obtained from Pierce Biotechnology Inc. (Rockford, IL). Substrates and inhibitors were obtained from Sigma Chemical Co. All other materials were of reagent grade.

**Phenols.** Total phenols were determined using the Folin−Ciocalteu reagent following a modification of the method reported by Singleton et al. (*29*). Gallic acid was used as the phenol standard.

**Carbohydrates.** Total carbohydrate content was determined using the phenol−sulfuric acid method as reported by Chaplin (*30*). Reducing sugar content was determined using dinitrosalicylic acid (*30*). Glucose was used as a carbohydrate standard.

**Protein.** Protein content was determined using the Bradford/Coomassie assay method following the manufacturer's instructions and the Lowry assay method (*31*) using BSA as a standard.

**Partial Purification of Tyrosinase.** Three hundred milligrams of crude WMT powder was resuspended in 40 mL of 10 mM sodium phosphate (pH 6.5) containing 1 mM PMSF. The enzyme was subjected to 35−65% saturation with ammonium sulfate. The 65% ammonium sulfate precipitate was dialyzed against 3 × 1 L of 5 mM sodium phosphate (pH 6.5) and then applied to a DEAE Sepharose CL-6B column (1.2 × 12.5 cm). Tyrosinase was eluted in a 0−0.5 M NaCl gradient. Fractions from the DEAE column containing tyrosinase activity were pooled and used as a source of partially purified WMT enzyme. This fraction still contained numerous proteins as indicated by SDS-PAGE.

Fifty milligrams of crude SMT powder was resuspended in 10 mL of 10 mM sodium phosphate (pH 6.5) containing 1 mM PMSF. The enzyme was subjected to 35−60% saturation with ammonium sulfate. The 60% ammonium sulfate precipitate was dialyzed and applied to a DEAE Sepharose CL-6B column as above. Pooled SMT tyrosinase fractions from the DEAE column were dialyzed and applied to a hydroxyapatite column (1 × 12.5 cm) equilibrated in 5 mM sodium phosphate (pH 6.9). Nonadsorbed tyrosinase fractions were pooled and made 1 M in NaCl. This sample was applied to a phenyl Sepharose column (ca. 5 mL) equilibrated in 20 mM sodium phosphate (pH 7.0) containing 1 M NaCl. The enzyme was eluted in a 1−0 M decreasing salt gradient. Fractions from the phenyl Sepharose column containing tyrosinase were pooled and used as a source of partially purified SMT tyrosinase. SDS-PAGE analysis still showed some low molecular weight proteins (12−14 kDa) and a few high molecular weight proteins (>55 kDa) present.

**Enzyme Assays.** Tyrosinase activity was determined using dopa as a substrate (*32*). Each 1 mL assay contained 5 mM dopa in 100 mM phosphate buffer (pH 6.5). Units of activity were defined as micromoles of dopachrome product formed per minute at 475 nm using a molar absorptivity value of 3600 $M^{-1}$ $cm^{-1}$. Dopa was dissolved in 0.5 mM phosphoric acid to make stock solutions of 10 mM. Laccase activity was determined using syringaldazine, ABTS, and tolidine as substrates as described earlier (*18, 19*).

Assays for $\beta$-glucosidase, $\beta$-galactosidase, $\beta$-mannosidase, $\beta$-xylosidase, and $\beta$-cellobiohydrolase were carried out using the corresponding $\beta$-*p*-nitrophenylglycosides ($\beta$-PNP-glucose, galactose, mannose, xylose, cellobiose) as substrates. Each 1 mL assay contained 50 mM sodium acetate (pH 5.0) and 4 mM *p*-nitrophenylglycoside. Aliquots were removed at various time intervals and added to 1 mL of 0.2 M sodium carbonate. Absorbance was measured at 400 nm, and 1 unit was defined as 1 $\mu$mol of product/min/mL using an absorptivity value of 18.1 $mM^{-1}$ $cm^{-1}$. Xylanase, cellulase, and chitinase activities were measured using 3-methyl-2-benzothiazolinone hydrazone (MBTH) for reducing ends of carbohydrates as described by Horn and Eijsink

(*33*) and using dinitrosalicylic acid (DNS) (*30*). Xylanase assays contained 0.5% beechwood xylan or oat spelt in 0.1 M sodium acetate (pH 5.0). Samples were rotated at room temperature and aliquots removed at various time intervals for reaction with MBTH. Absorbance was measured at 620 nm, and xylose was used as a standard. Chitinase assays contained 1 mg/mL chitin in 0.1 M sodium acetate (pH 5.0). Samples were rotated at room temperature and aliquots removed at various time intervals before further reaction with MBTH. *N*-Acetylglucosamine was used as a standard. Cellulase activity was determined using fibrous cellulose (type 50), carboxymethyl cellulose (CMC), and *p*-nitrophenylcellobioside (PNPC) as substrates. Cellulase assays contained 0.1% cellulose or CMC in 0.1 M sodium acetate (pH 5.0). Samples were rotated at room temperature and aliquots removed at various times for reaction with MBTH. In assays using MBTH, 1 unit of activity was defined as 1 $\mu$g of product/min/mL or 1 $\mu$mol of product/min/mL derived from the standard curves using glucose as a standard. PNPC cellulase assays were similar to those described for *p*-nitrophenyl (PNP) glycosidase assays above. Xylanase and mannanase assays were also carried out using methods described by Rescigno et al. (*22*).

**$I_{50}$ Determinations.** Various tyrosinase inhibitors (kojic acid, salicylhydroxamic acid, tropolone, methimazole, 4-hexylresorcinol, ammonium tetramolybdate) were dissolved in water to make stock solutions. Esculetin and quercetin were dissolved in 50% DMSO to make stock solutions. $I_{50}$ values were determined after various amounts of inhibitors were added to enzyme assays. Each 1 mL assay contained a fixed amount of enzyme, inhibitor, 5 mM dopa (final concentration), and 100 mM phosphate buffer (pH 6.5) (final concentration). $I_{50}$ values were extrapolated from plots of enzyme activity versus concentration of inhibitor.

**Electrophoresis, Western Blotting, and Isoelectric Focusing.** Native polyacrylamide gel electrophoresis (PAGE) and staining for tyrosinase activity was carried out as described earlier (*24*). Gels were also stained for monophenolase and diphenolase activity as described by Olianas et al. (*34*). $\beta$-Glucosidase activity staining was carried out as described by Kwon et al. (*35*). Xylanase activity staining was performed according to methods described by Schwarz et al. (*36*). SDS-PAGE was performed according to the Laemmli method in either 10 or 10−20% polyacrylamide gels. Gels were stained for protein using Coomassie Blue R250. Isoelectric focusing (IEF) was performed in precast agarose gels (pH 3−10) as described earlier (*32*). Proteins were stained with Coomassie Blue R250 after fixing, rinsing, and then drying gels onto the plastic backing. Tyrosinase activity was located by incubating gels in 100 mM phosphate buffer (pH 6.5) for 5 min and then incubating the gels in buffer containing 1 mM catechol and 2 mM MBTH. Gels were rinsed with water and dried onto their plastic backing. Western blotting was carried out using previous methods by the authors with antibodies prepared against commercial tyrosinase separated by preparative SDS-PAGE (*24*).

**Preparative SDS-PAGE and Protein Sequencing.** Approximately 2 mg of crude commercial mushroom tyrosinase powder was resuspended in 10 mM phosphate buffer (pH 6.5) and boiled for 2 min. One-fourth volume of 4× Laemmli sample buffer was added and the solution boiled again. Samples were applied to a 1 mm thick (10 × 10 cm) 11% SDS-PAGE gel. After electrophoresis was terminated, the gel was soaked in transfer buffer (10 mM CAPS, 10% methanol, pH 11) for 10 min before electrophoretic transfer onto ABI Protoblott PVDF membranes using the same buffer. After transfer, the membrane was stained briefly with Coomassie Blue R250 and destained extensively. N-Terminal protein sequencing of individual bands present on the PVDF membranes was carried out by the Michigan State University Macromolecular Structure Facility using standard Edman degradation procedures. Internal protein sequences of some bands on the PVDF membranes were determined after tryptic digestion of the band(s), HPLC separation of peptides, and N-terminal protein sequencing of selected peptides from the HPLC separation. Protein sequences were compared to proteins in the NCBI nonreductant database using BLASTP searches.

## RESULTS

Because of the continuous use of commercial MT preparations by various investigators, we decided to examine the composition of commercial MT with regard to tyrosinase and other biological material in these preparations. Sequencing, tyrosinase characterization, and glycosidase contamination studies were carried out using MT from Worthington because we have been using this source of MT for several years. All other studies used samples from Worthington and Sigma for comparison of MT from two commercial sources. In addition, a comparison of the crude Sigma powder versus a partially purified Sigma MT was used in some studies with inhibitors. Using dopa as a substrate, WMT and SMT showed different levels of tyrosinase activity per milligram of powder (**Table 1**). $K_m$ values, using dopa as a substrate, were reasonably similar for the two commercial preparations also (**Table 1**).

**Table 1.** Summary Characteristics of WMT and SMT Preparations[a]

| parameter | WMT | SMT |
|---|---|---|
| tyrosinase[b] (units/mg of powder) | 498 | 1700 |
| phenols ($\mu$g/100 $\mu$g of powder) | 2.8 | 2.6 |
| sugars ($\mu$g/100 $\mu$g of powder) | 50 | 40 |
| protein ($\mu$g/100 $\mu$g of powder) | 26 | 27 |
| native PAGE tyrosinase isoforms | 1 major<br>2 minor | 1 major<br>3−4 minor |
| IEF tyrosinase isoforms | 4 major<br>(p$I$ = 4.4−4.7) | 4 major<br>(p$I$ = 4.3−4.6) |
| SDS-PAGE tyrosinase subunit size (kDa) | 45 | 45 |
| $K_m$ dopa (mM) | 0.46 | 0.25 |

[a] Worthington mushroom tyrosinase (WMT, crude powder, lot 33H6588Q) and Sigma mushroom tyrosinase (SMT, crude powder, lot 105k7026) were characterized using parameters described under Materials and Methods. [b] Activity using dopa as a substrate for tyrosinase.

Both commercial MT sources showed similar levels of phenol content (**Table 1**), although we believe these values may be underestimated considering the brown color associated with the resuspended commercial powder. The brown material is presumably related to polymeric phenolic melanin-like material derived from the mushrooms or formed during preparation of the commercial enzyme. Some of the brown material is nondialyzable through 10 kDa dialysis membranes. In addition, this brown material spreads throughout column fractions during size exclusion chromatography. This brown material also forms a band near the dye front of native and SDS-PAGE. A UV−vis scan of MT powder resuspensions from 230 to 600 nm showed broad absorption from 300 to 400 nm with a peak absorption at approximately 340 nm in both MT preparations. The SMT preparation showed a more well-defined peak at 280 nm, whereas the WMT preparation showed a much broader peak at 280 nm (data not shown). The broad absorption from 300 to 400 nm is presumably related to the brown material because a partially purified tyrosinase isolated from these powders is nearly colorless and does not show large absorbencies at 340 nm (data not shown).

Both commercial MT preparations contained a large amount of carbohydrate material (**Table 1**). This material accounted for approximately 50% of the mass of the commercial MT powder. This carbohydrate material was also soluble in buffer solutions (pH 6−8) because dissolution of the powder was fairly rapid. We have noticed a precipitate that formed at pH below 5.5. This off-white precipitate did not contain tyrosinase activity and may contain some of the carbohydrate material. We also found that one lot of WMT (33E8802) contained approximately 12% reducing sugars using the DNS reagent (data not shown).

Using the Bradford/Coomassie protein assay as an index of protein content, the two commercial powders showed similar protein levels, approximately 30% of the total powder mass (**Table 1**). Using the Lowry assay, the protein content (13%)

was much lower in a sample that was precipitated with trichloroacetic acid before protein assays were carried out (data not shown). This suggests some material in the powder resuspensions may be interfering with some of the protein assays. On the basis of staining intensity of proteins in the commercial powders after SDS-PAGE (**Figure 1**) and comparison to a known amount of BSA, we believe the protein content to be roughly 10−20% of the total powder mass.

Both tyrosinase preparations showed one major tyrosinase isoform after native PAGE and staining for tyrosinase with catechol/dopa (**Table 1**; **Figure 1A**). However, the number, position, and intensity of the minor tyrosinase isoforms were not identical in the two MT preparations. All isoform staining was related to tyrosinase because addition of a tyrosinase inhibitor, salicylhydroxamic acid (SHAM), eliminated or reduced the staining intensity of all tyrosinase isoforms present (data not shown). IEF identified at least four major tyrosinase isoforms in both WMT and SMT powders after staining with catechol/MBTH (**Table 1**; **Figure 1B**). The major tyrosinase isoforms were spaced closely together, shared approximately equal staining intensity, and had p*I* values between pH 4.3 and 4.7. Many other minor tyrosinase isoforms with faint staining intensity were located at positions surrounding the major isoforms. Protein staining of these IEF samples showed the presence of many proteins unrelated to tyrosinase. These proteins had p*I* values lower and higher than those of MT. On closer inspection, some of these proteins located in the pH range of 4.4−4.7 did not correspond to bands of tyrosinase activity staining.

Both commercial sources of MT showed many bands of proteins after SDS-PAGE (**Figure 1C**). Five to seven major protein bands were observed in the SMT powder, and five to six major protein bands were present in the WMT powder. Many minor bands of protein were also present, and we estimated they numbered from 10 to 20. These bands were too faint or too closely spaced together to be seen well in **Figure 1**. The major bands showed molecular masses in the range of 14−18, 26, 45, and 55−66 kDa. The protein band(s) at 45 kDa cross-reacted with antibodies to denatured mushroom tyrosinase (data not shown).

In addition to tyrosinase, laccase and several glycosidase activities were detected in commercial WMT (**Table 2**). Laccase activity was detected in both crude commercial MT powders and partially purified MT using ABTS and tolidine but not in syringaldazine as substrates. We were also able to detect $\beta$-glucosidase, $\beta$-galactosidase, and $\beta$-xylosidase but not $\beta$-mannosidase activities in both crude powders and partially purified tyrosinase fractions. $\beta$-Glucosidase activities appeared to be greater than those of the other glycosidases on the basis of enzyme assays and time course studies. Cellulase, chitinase, xylanase, and mannanase activities were also detected in the crude MT powder resuspensions but not in partially purified samples. On the basis of cellulase substrates (fibrous cellulose, CMC, PNPC), it appeared that both endo- and exoglucanase activities were present in the crude commercial MT. After one particular lot of WMT was subjected to non-denaturing PAGE and stained for tyrosinase, $\beta$-glucosidase, and xylanase, several isoforms of each enzyme were detected (**Figure 2**, data not shown). Staining for the monophenolase activity of tyrosinase showed one major and one minor isoform. Staining for the diphenolase activity of tyrosinase showed one major and two minor isoforms. Three $\beta$-glucosidase isoforms were detected, none of which were located at the same position as tyrosinase. Staining for $\beta$-glucosidase activity results in gray-black bands of staining. These bands appear above tyrosinase. A different color deposition occurs in the tyrosinase band, probably because of nonspecific binding of ferric chloride to tyrosinase. This nonspecific binding results in an apparent dark area below the glucosidase bands in black and white photodocumentation in

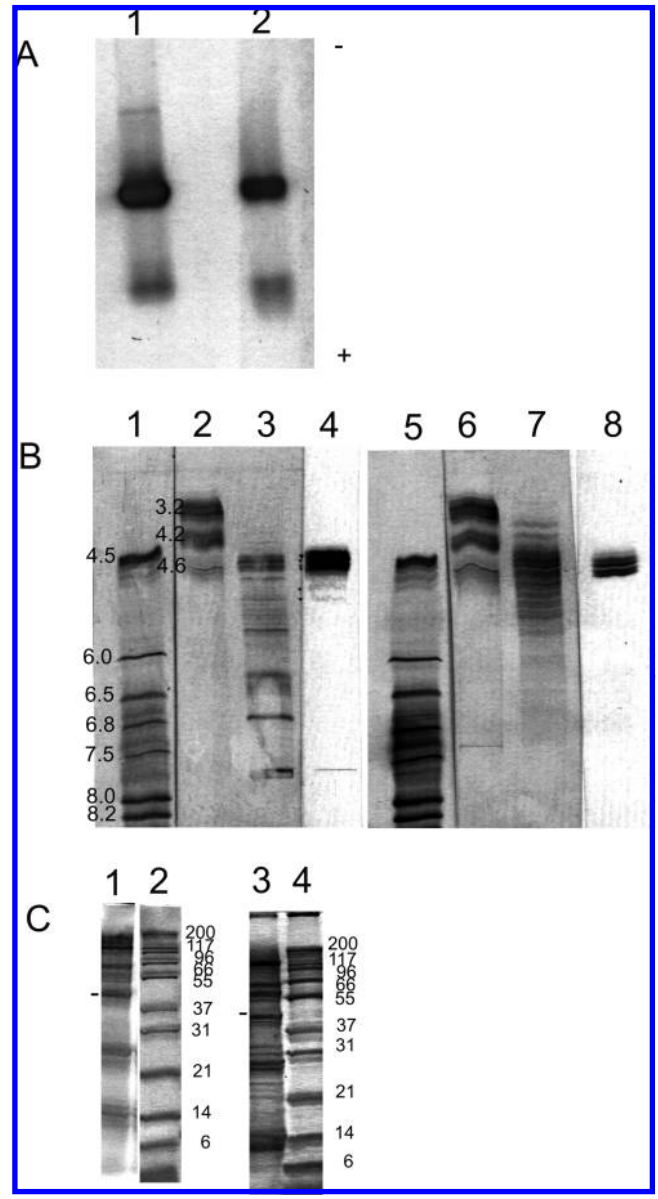


**Figure 1.** Native PAGE, IEF, and SDS-PAGE of WMT and SMT isoforms. (**A**) Native PAGE, IEF, and SDS-PAGE were carried out as described under Materials and Methods. Tyrosinase isoforms were located by staining native polyacrylamide gels in a catechol/dopa solution plus or minus SHAM (300 $\mu$M) as an inhibitor. Approximately 3.5 $\mu$g of SMT (lane 1) and WMT (lane 2) powder was applied per lane. (**B**) IEF gels were stained for protein using Coomassie Blue or for tyrosinase using catechol/MBTH. IEF standards were from Bio-Rad (lanes 1 and 5) and from Sigma Chemical Co. (lanes 2 and 6). Approximately 30 $\mu$g of SMT powder (lane 3) and 40 $\mu$g of WMT powder (lane 7) were applied and stained for protein. Approximately 2.5 $\mu$g of SMT powder (lane 4) and 2.5 $\mu$g of WMT powder (lane 8) were applied and stained for tyrosinase activity. Isoelectric points (p*I* values) are listed to left of protein standards. (**C**) Approximately 40 $\mu$g of SMT powder (lane 1) and approximately 60 $\mu$g of WMT powder (lane 3) were applied to 10−20% SDS−polyacrylamide gels. Protein standards from Invitrogen were used as markers and for molecular weight estimations (lanes 2 and 4). Hash marks indicate position of 45 kDa protein/tyrosinase band.

**Table 2.** Summary of Enzyme Activity in WMT Preparations[c]

| enzyme | CWMT (total units) | PPMT (total units) | % activity in PPMT |
|---|---|---|---|
| tyrosinase | | | |
| dopa | 444 | 232 | 52 |
| laccase | | | |
| tolidine | 30 | 12 | 40 |
| ABTS | 0.76 | 0.36 | 47 |
| syringaldazine | ND[d] | | |
| $\beta$-glucosidase | 100 | 0.79 | 0.79 |
| $\beta$-galactosidase | 2.64 | 0.105 | 4 |
| $\beta$-mannosidase | ND | ND | |
| $\beta$-xylosidase | 8.48 | 1.2 | 14 |
| cellulase | | | |
| type 50 cellulose[b] | 0.224 | ND | |
| CMC[b] | 0.276 | ND | |
| PNPC | 8.4 | ND | |
| chitinase | | | |
| crab shell, chitin[b] | 0.052 | ND | |
| xylanase | | | |
| xylan, beechwood[b] | 0.116 | ND | |
| xylan, birchwood[c] | 7.06 | | |
| xylan, oat spelt[c] | 3.81 | | |
| mannanase | | | |
| galactomannan, locust bean[c] | 0.56 | | |

[a] Worthington mushroom tyrosinase (CWMT, lot 36E8802) was assayed for laccase and various glycosidases as described under Materials and Methods. Partially purified Worthington MT (PPMT) was obtained after a 35−60% ammonium sulfate precipitation and DEAE ion exchange chromatography. [b] Assays carried out using the MBTH assay. [c] Assays carried out using the DNS assay. [d] ND, not detectable.

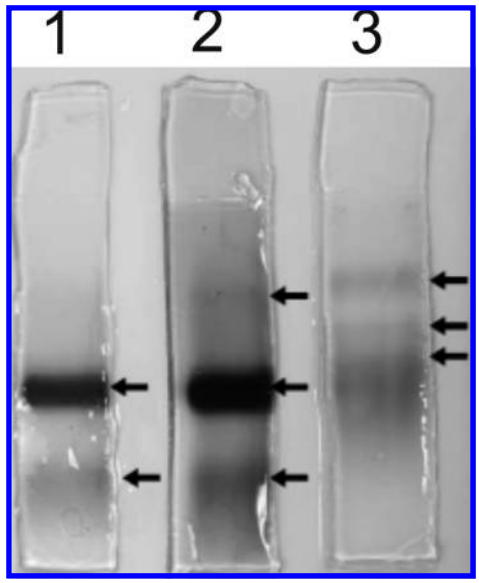


**Figure 2.** Tyrosinases and glycosidases in WMT. Native PAGE was carried out as described under Materials and Methods using 10% polyacrylamide gels. Approximately 200 $\mu$g of the original powder was applied. The gel was cut into sections and stained for monophenolase activity of tyrosinase (lane 1), diphenolase activity of tyrosinase (lane 2), and $\beta$-glucosidase (lane 3). Arrows point out major staining areas of tyrosinase in lanes 1 and 2 and $\beta$-glucosidase activity in lane 3. Nonspecific staining of tyrosinase is located beneath glucosidase staining in lane 3.

**Figure 2**. One band of xylanase activity was detected. The location of this band of staining did not match either tyrosinase or $\beta$-glucosidase enzyme staining (data not shown).

Two different WMT commercial preparations (33H6588Q and 36E8802) were subjected to preparative SDS-PAGE, transfer onto PVDF membranes, and protein sequencing. The results of these are shown in **Table 3**. Only the major protein bands were selected for protein sequencing. Of all the sequences obtained, only three sequences were identified and matched existing protein sequences in databases [initially reported in Rescigno et al. (*22*)]. These three sequences corresponded to *A. bisporus* tyrosinase (*37*), a putative *A. bisporus* mannanase (*38*), and *A. bisporus* lectin (*39*). Ten peptide sequences were not identified at this time.

**Table 3.** N-Terminal and Internal Peptide Sequences from SDS-PAGE of WMT[a]

| band | mol mass (kDa) | peptide/protein sequence |
|---|---|---|
| 5 | 45 | N-terminal blocked |
| | | tryptic digest |
| | | peak 10, T A D D Q A E G I O |
| | | peak 16, D P T V Y Q (A) (F); |
| | | E Q D T G L I P G Q; |
| | | L N I L D F V K (*Agaricus* AbPPO2, tyrosinase)[b] |
| | | peak 28, D I K I − Q Y; |
| | | E V Y F T V L − V; |
| | | F L E A I S Q Y P N I |
| 6 | 26 | A I D F P F A (A) E D V V Q T |
| | | A I D F P F A A E D |
| 7 | 18 | A I V V (V) X D V (T) |
| | | tryptic digest |
| | | peak 11, A G N A L D L S G V D D[b] |
| | | (putative *Agaricus* mannanase) |
| 8 | 16 | T Y T I S I R V Y Q (*Agaricus* lectin)[b] |
| | | T Y T I S I A V Y Q |
| 9 | 12−14 | A T N S L I L I I Q |
| | | (V) I G T G T I F F |

[a] Preparative SDS-PAGE, transfer to PVDF membranes, and protein sequencing of WMT were carried out as described under Materials and Methods. Band number refers to position of protein-stained bands in SDS-PAGE. A corresponding molecular weight for each band is given beside band number. Peptides from tryptic digests of specific bands are identified by HPLC peak number. [b] Initially reported in Rescigno et al. (*22*).

Two N-terminal protein sequences in the range of 12−14 kDa were found (**Table 3**). Presumably, these correspond to two different proteins and neither of these matched sequences to any proteins in the databases. The band at 16 kDa was identified as *Agaricus* lectin from its N-terminal protein sequence (accession no. AAA85813; *39*39). Ten amino acids of 10 matched a corresponding sequence for this lectin. Apparently this band contained a single protein because no other N-terminal sequences were obtained. N-Terminal sequencing of the 18 kDa protein generated a sequence with no similarity to any proteins in the databases. However, a tryptic digest of this protein (HPLC peak 11) generated a sequence that matched a putative *Agaricus* mannanase (accession no. CAD58856; *38*38). Twelve of 12 amino acids matched the corresponding sequence for this protein. The 26 kDa protein generated an N-terminal protein sequence that had no matches in the databases. Only one peptide sequence was obtained, suggesting this band contained a single protein component.

Two sequencing attempts of the 45 kDa protein band were unable to generate a peptide sequence, suggesting that the N terminus was blocked. Tryptic digests and separation by HPLC generated multiple peptide peaks with identifiable and unidentifiable protein sequences. This suggests that the 45 kDa protein band probably contained a major N-terminal blocked protein(s) and several minor proteins that may or may not have an N terminus blocked. One peptide (HPLC peak 10) contained a peptide sequence of an unknown protein. Another peptide (HPLC peak 28) contained three peptide sequences, none of which matched any protein sequences in the data banks. Another peptide (HPLC peak 16) contained three peptide sequences. Two

**Table 4.** $I_{50}$ Values for Tyrosinase Inhibitors Using WMT and SMT[a]

| | $I_{50}$ value ($\mu$M) | | |
|---|---|---|---|
| | WMT | SMT | |
| inhibitor | crude powder | crude powder | partially purified |
| kojic acid | 45 | 30 | 25 |
| salicylhydroxamic acid | 0.8 | 1.2 | 0.9 |
| esculetin | 210 | 225 | |
| tropolone | | 1.3 | 1.7 |
| methimazole | | 40 | 47 |
| 4-hexylresorcinol | | 18 | 2.5 |
| ammonium tetrathiomolybdate | | 28 | 7.5 |

[a] Crude commercial powders from WMT and SMT were used to determine $I_{50}$ values as described under Materials and Methods. A partially purified form of SMT (see Materials and Methods) was also used for $I_{50}$ determinations.

of these sequences were not identified in protein data banks, whereas one peptide (LNILDFVK) matched the protein sequence for *A. bisporus* tyrosinase (PPO2, accession no. CAA11562 *37*). Seven of eight amino acids matched this sequence. Because the unknown peptide sequences in the SDS-PAGE band at 45 kDa did not match more of the known *Agaricus* tyrosinase sequence, this strongly suggests that the 45 kDa band does not contain a single protein and is composed of proteins with similar molecular weights.

Because many investigators use commercial mushroom tyrosinase to screen compounds for tyrosinase inhibitors and to obtain $I_{50}$ or $K_i$ values for these inhibitors, we decided to compare a few inhibitory characteristics of the two commercial MT powders (**Table 4**). Because most of the investigators use crude SMT in their inhibition studies, a partially purified SMT was also prepared for comparison to tyrosinase in the corresponding crude commercial powder. Similar $I_{50}$ values for kojic acid, salicylhydroxamic acid, esculetin, tropolone, and methimazole were noted when the two commercial tyrosinase preparations were compared or when crude versus partially purified tyrosinase preparations were compared. In contrast, we found a significant difference in $I_{50}$ values for 4-hexylresorcinol (4HR) and ammonium tetrathiomolybdate (ATTM) for the crude SMT versus the partially purified SMT. Reductions in $I_{50}$ values of approximately 6- and 4-fold for 4HR and ATTM were noted, respectively. We also observed significant inhibition of SMT by ethanol and could not test inhibition of 4,4′-dihydroxybiphenyl and quercetin as inhibitors because of solubility problems associated with the inhibitors and inhibition by ethanol (data not shown).

## DISCUSSION

It is clear that commercial MT preparations contain material that is nonprotein in nature. The sugar content, which we assume to be a polymer, is relatively high and is the major contaminant in the commercial powders. During preparation of the commercial mushroom tyrosinase, it is likely that the major soluble carbohydrates, mannitol and trehalose, of *Agaricus* (*40*) are absent in the final commercial MT powder. The major polymeric cell wall carbohydrates in *Agaricus* are chitin and glucans, whereas the nonstructural polysaccharides are probably glycogen-like (*41*). These polymeric carbohydrates may be the material we are detecting in the phenol−sulfuric acid method because glycosidic linkages should be hydrolyzed by the sulfuric acid in this assay. It is not unlikely that these polymers could trap, bind to, or react with tyrosinase and/or its substrates or inhibitors.

The brown color associated with the commercial MT powder is probably melanin-like polyphenolic material. Although the phenol content appeared to be small (ca. 3%), we measured only phenols capable of reacting with Folin's reagent. We did not try to separate simple phenols from polymeric phenols, although we have observed many times that some of this brown material is nondialyzable through 10 kDa membranes. This brown material is also present throughout most of the fractions during size exclusion chromatography, suggesting a large variation in polymer sizes and/or adsorption onto chromatographic resins. It also appears that some of this material is negatively charged because it binds to DEAE cellulose at pH 7.0 and migrates to the dye front in native and SDS-PAGE. Because of its anionic and aromatic phenol-like characteristics, it is likely that this material could exhibit chemical reactivity and react with other biological material. This polyphenolic material may also be able to adsorb various types of biomolecules (e.g., enzymes, proteins) and organic compounds (e.g., tyrosinase substrates, inhibitors) because of its ionic and aromatic characteristics.

Commercial tyrosinase preparations from *A. bisporus* have different isoform compositions compared to crude extracts from *A. bisporus* (*28*). In this study several active tyrosinase isoforms were present in the both crude commercial mushroom tyrosinase preparations and were similar but not identical to that reported earlier (*42*). Tyrosinase isoforms from both commercial preparations in this study differed slightly in their p*I* forms but did not differ appreciably in subunit size. Even though the tyrosinase isoform composition appeared to be similar, the two commercial MT preparations showed much different activities based on enzyme units per dry powder weight.

Many of the studies using mushroom tyrosinase to screen inhibitors have determined $K_i$ or $I_{50}$ values for these inhibitors and have used the inhibitor kojic acid as a reference for comparison. Of the papers we examined, and those examined by Marrero- Ponce et al. (*17*), that used kojic acid, $I_{50}$ values for this inhibitor ranged from approximately 3 to >300 $\mu$M. This in itself is quite remarkable and illustrates the potential problems of estimating $I_{50}$ values for MT. Even though we were aware of these problems, we decided to compare $I_{50}$ values for a few tyrosinase inhibitors reported in the literature. Our $I_{50}$ values for kojic acid (25−45 $\mu$M) were similar to one another whether the crude SMT/WMT powders or a partially purified SMT was used. Our $I_{50}$ values for SHAM and tropolone using crude or partially purified MT were slightly higher than that reported by Kahn (*43*) and Kahn and Andrawis (*44*). In contrast, our $I_{50}$ values for methimazole (40−50 $\mu$M) were lower than the estimated 200−300 $\mu$M reported by Andrawis and Kahn (*45*).

Park et al. (*46*) reported an $I_{50}$ value of 10.7 $\mu$M for ammonium tetrathiomolybdate (ATTM) using crude commercial SMT. We found a higher $I_{50}$ value (28 $\mu$M) for ATTM using the crude SMT, but a lower $I_{50}$ value (7.5 $\mu$M) using a partially purified tyrosinase. This anomaly between two different samples of crude SMT and crude versus partially purified SMT is not easy to explain. Our $I_{50}$ values for 4-hexylresorcinol (2.5, 18 $\mu$M) were much higher than that of 0.85 $\mu$M reported by Chen et al. (*47*), even though our crude SMT gave a higher $I_{50}$ value than the partially purified MT (18 vs 2.5 $\mu$M, respectively). Once again, use of crude SMT preparations resulted in higher $I_{50}$ values than those of partially purified SMT. Even though $I_{50}$ determinations are assay dependent, our data suggest that some compounds may display different inhibition characteristics depending on the source and state of purity of MT. This observation has not been considered in many of the inhibition studies related to MT. In addition, it is not prudent to extrapolate

data using mushroom tyrosinase to that of mammalian tyrosinase in inhibitor studies for skin-whitening agents. Jacobsohn and Jacobsohn (*48*) and Galindo et al. (*49*) have already reported that mushroom tyrosinase behaves differently from mammalian tyrosinase with regard to specific inhibitors and substrates.

We estimated that there were at least 15−30 proteins present in commercial WMT preparations visible by Coomassie Blue staining after SDS-PAGE. Five to seven major protein-stained bands were relatively abundant. Our N-terminal analysis of some of these major proteins indicated the presence of at least 11 peptide/protein components (**Table 3**). N-Terminal analysis did show that although some bands appeared to be a single protein by SDS-PAGE staining, they really contained a mixture of proteins with similar sizes (see **Figure 1** and **Table 3**).

The major protein(s) in our WMT 45 kDa band had a blocked N terminus. Schurink et al. (*50*) recently reported that a 49 kDa form of purified SMT had a blocked N terminus. We do not know the total number of proteins in the 45 kDa band, but they must number greater than two, depending on how many unknown sequences correspond to different proteins (**Table 3**). Of the protein(s) clustered in that 45 kDa band, only one of these matched a known tyrosinase sequence. Seven of eight amino acids from the WMT sequence (LNILDFVK) matched the SMT sequence (LNIVDFVK) reported by Schurink et al. (*50*). This sequence corresponded to a region near the N terminus of the deduced protein sequence of *A. bisporus* PPO2 tyrosinase (ca. 64 kDa) that was reported to be an inducible tyrosinase and not the constitutively expressed PPO1 tyrosinase (*37*). All of the above indicate that the 45 kDa form is derived from the N-terminal end of a processed tyrosinase.

We found a peptide sequence (ATNSLILIIQ) for proteins in the 12−14 kDa range (**Table 3**). This sequence did not match any protein sequences in the data banks or sequences related to *Agaricus* tyrosinases PPO1 or PPO2. Schurink et al. (*50*) also found a similar protein sequence (ATNSGTLIIFD) in the 12−14 kDa protein range in their purified SMT "L subunit" that did not match the sequences of mushroom tyrosinases PPO1 or PPO2. These data suggest that these 12−14 kDa peptide/proteins (L subunits, *51*) are not derived from tyrosinase at all. More likely, these sequences correspond to some other protein(s) besides tyrosinase.

Gene sequences and in vivo proteolysis have established that the native *Agaricus* tyrosinase is a monomer of 64 kDa that can be rapidly processed to a 45 kDa fragment (catalytically active) and an inactive 15 kDa fragment by C-terminal processing (*3, 23, 50*). This finding is not without precedent with regard to tyrosinase. In vitro C-terminal processing of broad bean tyrosinase (60 kDa) was shown to generate a 42 kDa protein and 12, 14, and 18 kDa proteins, depending on the protease used (*52*). The 12−18 kDa broad bean proteins were difficult to visualize on SDS gels and were later degraded into smaller peptides by proteases in vitro, and presumably in vivo. Likewise, the C-terminal 15 kDa fragments of mushroom tyrosinase may be degraded into much smaller peptides (<10 kDa) during preparation of the commercial tyrosinase, making them difficult to detect by SDS-PAGE. The fact that the sequences of the 12−14 kDa proteins did not match tyrosinase supports this hypothesis.

Of the other two major proteins identified by N-terminal sequencing, one of them corresponded to *A. bisporus* lectin [ABL; Carrizo et al. (*39*)]. This lectin binds carbohydrates containing Gal$\beta$1−3GalNac (Thomsen−Friedenreich disaccharide glycotope), has a subunit size of 16 kDa (similar to that in this paper), and is found as a tetramer of 64 kDa. ABL isoforms have p*I* values in the range of pH 4−6.5. Some of these lectin characteristics are very similar to those of mushroom tyrosinase with regard to native size and p*I*. This lectin has the potential to bind phenolic glycosides containing the above carbohydrate composition. However, these sugar groups are probably not found in typical phenolic glycosides isolated from plants and fungi. In addition, if the Gal$\beta$1−3GalNac sequence is found in antibodies, the *Agaricus* lectin could bind to them and potentially interfere with immunological assays for detection of tyrosinase. In any case, this protein is one of the major contaminants in the commercial MT preparations.

A second protein we identified by sequencing, designated as a putative mannanase by Morales and Thurston, is more likely a glycosidase of some type (*53*). Extracellular glycosidases are excreted by *A. bisporus* under a variety of conditions. Some of these include xylanases, mannanases, cellobiohydrolases, endoglucanases, and $\beta$-glucosidases (*53–57*). Several of these enzymes were detected in our preparation of crude and partially purified WMT. Rescigno et al. (*22*) have been able to detect xylanase, $\beta$- xylosidase, and $\beta$-glucosidase activities in commercial SMT preparations. These glycosidases may account for some of the numerous minor protein contaminants we have observed in SDS-PAGE of WMT and SMT. More importantly, these glycosidases, and especially $\beta$-glucosidases, could potentially hydrolyze phenolic glucosides used as tyrosinase substrates or inhibitors. Recently, Rescigno et al. (*22*) have shown that $\beta$-glucosidase treatment of esculin, a phenolic glycoside that does not inhibit MT, causes MT inhibition. Presumably this occurs by conversion to esculetin, a phenolic MT inhibitor. It is difficult to estimate glycosidase effects on tyrosinase activity or tyrosinase inhibitor screening because their presence has been of little concern to those working with commercial MT preparations. The presence of glycosidases, along with other enzymes, proteins, carbohydrates, and phenolic material, may affect results and interpretations when using commercial tyrosinase in various types of studies.

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