The Multiple Forms of Mushroom Tyrosinase

PURIFICATION AND MOLECULAR PROPERTIES OF THE ENZYMES

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We have undertaken the purification of mushroom tyrosinase to obtain homogeneous enzyme for a study of its mechanism, which is unknown. Purified mushroom tyrosinases have already been reported (1–9). Some of these preparations were homogeneous, or almost homogeneous, by either electrophoresis or ultracentrifugation; one was homogeneous by both criteria (3, 9). However, there are discrepancies among them. According to one report, the molecular weight is 34,000 (3); according to others, it is close to 100,000 (2, 4–6, 9). The copper which the enzyme contains is either partially (6) or entirely cuprous (10). Moreover, if tyrosinase is a single protein able to catalyze o-hydroxylation of monophenols and dehydrogenation of o-diphenols, the ratio of these two kinds of activities, often referred to as cresolase and catecholase activities, depends on the method of purification (2).

In 1949, Mallette and Dawson (2) obtained mushroom tyrosinases with different physical and enzymic properties which they ascribed to chemical or physicochemical changes in an original single enzyme during the purification process. Their enzymes contained different amounts of copper and different ratios of cresolase to catecholase activity, but they could not be separated from one another by ultracentrifugation or electrophoresis. Recently, Smith and Krueger (8) showed that five tyrosinase activities can be separated from mushroom extracts by chromatography on hydroxylapatite columns. One of their active fractions moved as a single substance during starch gel electrophoresis, but the other fractions were heterogeneous.

In this study, we have confirmed the multiplicity of mushroom tyrosinase. Using classical procedures and an efficient preparative electrophoresis followed by chromatography on hydroxylapatite, we obtained four proteins: the α-, β-, γ-, and δ-tyrosinases, three in homogeneous form. In this article, we describe the preparation of the enzymes and some of their enzymic, chemical, and physical properties.

EXPERIMENTAL PROCEDURE

Materials—Commercial Agaricus bispora was used as starting material. This is probably identical with the commercial mushrooms used as source material in other studies and identified as Psalliota campestris or Agaricus campestris. p-Cresol and catechol were purified from Eastman Kodak Company reagents.

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Sodium dodecyl sulfate was recrystallized from ethanol from Matheson, Coleman and Bell, Inc., reagent. Hydroxylapatite was prepared according to the method of Tiselius (11). All other materials were best commercial grade, and deionized, distilled water was used throughout the study.

Estimation of Enzymic Activities—Although the assay conditions and units of monophenol hydroxylase and o-diphenol dehydrogenase activities were defined some years ago (12, 13), modifications and novel assay procedures have been introduced. The units recommended by the Commission on Enzymes ought to be generally adopted, but we have in this paper retained the classical units for such comparisons as are possible. We determined activity towards p-cresol and catechol according to the method of Gregg and Nelson (12), using, however, smaller Warburg flasks and smaller total volumes, i.e. 3.2 ml rather than 8.0 ml, and making the appropriate correction. The chromometric unit of Miller, Mallette, Roth, and Dawson (13) was also used where noted. Activity toward L-tyrosine and DL-3,4-dihydroxyphenylalanine was determined manometrically with 1 mg of substrate per 3.2 ml, total volume, at 25°, the unit being the amount of enzyme required to catalyze the consumption of 1 μl of O2 per minute (cf. Lerner (14)).

Protein Estimation—For first approximations, we used the spectrophotometric determination of Eiger and Dawson (15). However, it became obvious with our more purified fractions that a large discrepancy exists between this method and a biuret determination based on a commercial, stabilized globulin standard, Lab-Trol (Dade Reagents, Inc.). The biuret method gave from 54 to 68% of the values obtained spectrophotometrically at this stage.

Total Copper Estimation—These were performed by the method of Stark and Dawson (16). To liberate the maximal amount of copper from the purified samples, 0.4 N rather than 0.2 N HCl was required. All determinations were made in duplicate, and calculations were based on a standard curve determined simultaneously under identical conditions.

Cuprous Copper Determinations—We used several modifications of cuprous copper determinations (10, 17, 18). Our experience with these determinations is discussed at greater length under "Results."

Cupric Copper Determination—Cupric copper was determined by electron spin resonance spectroscopy. We used a Varian V-4500 spectrometer with 100-kc field modulation. Samples and standard (2 mM CuSO4 in 20 mM EDTA) were examined under exactly the same conditions, usually at −165°. The
spectra were recorded as derivatives of absorption with respect to field strength; the double integral of the unknown and standard were determined by graphical methods and compared. This procedure applied to other copper proteins in our laboratory has been shown to be accurate to within 2% at 0.1 mM Cu++, and measure cupric copper only. The difference between this estimation and total copper gives an independent measure of cuprous copper.

Amino Acid Analyses—Exploratory analyses were made with the Beckman/Spinco model 120 automatic analyzer, after 20 hours of hydrolysis at 110° by 0 M HCl, with the reaction tube sealed under vacuum (19–20). Tryptophan was determined separately by the method of Goodwin and Morton (21).

Free Boundary Electrophoresis—These determinations were made with the Spinco instrument, and the β- and γ-enzymes in phosphate buffer, pH 6.8, ionic strength, 0.1.

Ultracentrifugal Analysis—Experiments to test the homogeneity of our tyrosinases were performed, with the kind help of Dr. S. Inouye, with the Spinco model E centrifuge at 45,000 r.p.m., and solutions containing from 3 to 5.4 mg of protein per ml (biuret) in 5 mM Na2HPO4.

A preliminary study of the behavior of an homogeneous tyrosinase (2) in the presence of increasing amounts of sodium dodecyl sulfate was made by R. D. Wade, using a similar instrument at 59,780 r.p.m., and a double sector, capillary-type, synthetic boundary cell. The protein concentration was 2.68 mg per ml in 5 mM Na2HPO4. The comparison solution contained the same amount of sodium dodecyl sulfate as the enzyme solution in each experiment.

Molecular Weight Determinations—These measurements were made with homogeneous β- and γ-tyrosinases with sedimentation equilibrium with the short column multichannel cell described by Yphantis (22). Values of the initial concentrations were obtained from synthetic boundary cell runs. A partial specific volume of 0.75 ml was assumed for the proteins.

Continuous Flow Electrophoresis—We used the refrigerated Beckman/Spinco model CP continuous flow electrophoresis apparatus. The enzyme extracts were always introduced at the cathodic upper side of the curtain. Preliminary experiments showed that most of the components in the extracts migrated towards the anode. Similar distributions were obtained with various buffers in the pH range 5.9 to 8.7, ionic strength, 0.02, and we adopted for routine use, Tris-HCl, pH 7.69, ionic strength, 0.02, with a current of 20 to 22 ma at 340 to 360 volts, the buffer flow and sample feed rates being dependent on protein concentration (no more than 35 mg per ml for crude extracts). The enzyme localization was determined by oxidation of tyrosine, p-creosol, or catechol. Protein distribution was determined spectrophotometrically at 280 mp, after appropriate dilution and correction for the presence of pigment if necessary (15).

Chromatography on Hydroxylapatite—The gel was prepared according to Tiselius (11) in 0.001 M phosphate buffer, pH 6.8. A column 2.2 cm in diameter and 25 cm long was three-fourths filled with gel and washed with buffer. The sample, containing no more than 200 mg of protein in no more than 15 ml of buffer, was introduced, absorbed, and covered with a filter paper disk, then washed twice with small amounts of buffer. An approximately linear gradient of buffer concentration, from 1 mM to 50 mM, was established by a two-vessel syphon, and the final concentration was maintained until the third enzyme fraction (γ-tyrosinase) was eluted. A new gradient from 0.05 M to 0.2 M was then established. During the separation, buffer flow was controlled by a Milton Roy Minipump, usually at 4 ml/30 minutes, this being the volume collected in each tube of an automatic constant interval collector. The optical transmittance at 250 mp of the effluent was recorded continuously; absorbancies of the effluent fractions of interest were determined later.

Concentration of Dihide Enzyme—It was often necessary to concentrate the dihide enzyme solutions obtained from continuous flow electrophoresis, chromatography, or after dialysis of ammonium sulfate fractions of crude extracts. In the early stages, Carbowax 4000 was generally used around a dialysis bag containing the enzyme, but this was avoided in later steps. If the enzyme were not too dilute and already somewhat purified, it was brought to 0.7 saturation with ammonium sulfate, the precipitate was redissolved in a minimal volume, and the solution was dialyzed against any desired buffer. In case of higher dilutions of purified enzymes (for example, after chromatography), the dialysis bag containing enzyme was immersed in sucrose powder, in accordance with a suggestion of Dr. N. H. Horowitz, until a maximal decrease of volume had taken place. The solution was then dialyzed against buffer, and passed through a second sucrose cycle. The final dialyses against buffer were exhaustive in the case of the purified last stage enzyme.

Extraction of Tyrosinase—Fresh white mushrooms were frozen at −20° at least 1 day before extraction. We will describe now the first steps of enzyme preparation, modified from procedures reported by other investigators in the light of observations made in this laboratory over the last several years.

Step 1: Frozen mushrooms, 3 kg, were homogenized in 4 liters of acetone at −20° in a large Waring Blender, for no more than 1 minute. The homogenate was filtered through a 40-cm Buchner vacuum filter covered by a large nylon fabric cap on which was powdered Dry-Ice, placed in contact with the pulp as soon as most of the acetone phase had been sucked away. The whole process was repeated with the partially frozen, once-extracted pulp.

Step 2: The pulp obtained after the second dehydration was homogenized with 30% acetone in water (volume for volume) at 0° for 2 to 3 minutes in the same Waring Blender. We used 3 liters of liquid per kg of starting mushrooms. The mixture was centrifuged at 6000 r.p.m. in six 390-ml cups of the refrigerated Servall model RC-2 centrifuge. It is convenient to use two centrifuges when large quantities of mushrooms are being worked up.

Step 3: To the supernatant aqueous acetone extract obtained in the last step, 1.5 volumes of acetone at −20° were added. The mixture was allowed to settle in a freezer at −20°; most of the clear supernatant fluid was decanted and discarded, the remainder was centrifuged as in Step 2, and the precipitate was dissolved quickly in ice-cold water. Approximately 450 ml were used for the precipitate from a 6-kg extraction. This extract was then made 1% in calcium acetate by slowly adding a 10% calcium acetate solution. (Addition of calcium acetate was finally shown to give no increase in specific activity, at least as described here, although other investigators apparently derived some advantage from its use.) The turbid mixture was frozen at −20°. It was
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TABLE I
Purification data for Preparation J

<table>
<thead>
<tr>
<th>Stage</th>
<th>Protein total mg</th>
<th>Specific activities toward units/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>4. (NH₄)₂SO₄, 0.35 to 0.73 saturated precipitate</td>
<td>4600</td>
<td>Catechol 130, p-Cresol 25, Tyrosine 122</td>
</tr>
<tr>
<td>5. Refractionation at saturation 0.30-0.36</td>
<td>90</td>
<td>14</td>
</tr>
<tr>
<td>0.36-0.41</td>
<td>214</td>
<td>46, 15, 102</td>
</tr>
<tr>
<td>0.41-0.46</td>
<td>954</td>
<td>355, 33, 224, 205, 550</td>
</tr>
<tr>
<td>0.46-0.52</td>
<td>808</td>
<td>300, 30, 250, 200, 580</td>
</tr>
<tr>
<td>0.52-0.60</td>
<td>680</td>
<td>10, 8, 34</td>
</tr>
<tr>
<td>0.60-0.66</td>
<td>215</td>
<td>8, 20, 30</td>
</tr>
<tr>
<td>6. Lead subacetate-treated 0.41-0.46 fraction</td>
<td>500</td>
<td>46, 245</td>
</tr>
<tr>
<td>7. Continuous flow electrophoresis of previous samples</td>
<td>413</td>
<td>37</td>
</tr>
<tr>
<td>8. Hydroxylapatite chromatography, concentrated fractions</td>
<td>255</td>
<td>70, 890</td>
</tr>
</tbody>
</table>

* Starting from 32 kg of mushrooms. See "Results."

† Spectrophotometric determination of protein according to Eiger and Dawson (15). The values obtained with the biuret method listed for the purified enzymes (Steps 7, 8) were about 64 to 68% of those given by the spectrophotometric method.

‡ Chronometric assay of Miller, Mallette, Roth, and Dawson (13).

§ Manometric assay according to Gregg and Nelson (12). See "Experimental Procedure."

Enzyme preparation always followed the four steps described in "Experimental Procedure." Subsequent steps were carried out in different sequences from one preparation to another, and comments on them are given below. Purification data for our last preparation, J, are given in Table I. Data corresponding to the final step only for the two preceding preparations, F and M, are given in Table II.

**Ammonium Sulfate Fractionation (Step 5)**—The concentrate obtained in Step 4 contained approximately 10 mg of protein per ml. It was subfractionated with neutral saturated (NH₄)₂SO₄ at 0°. Successive precipitates were collected by centrifugation 2 hours after each addition of salt, redissolved in cold water, and dialyzed against 5 mm Na₂HPO₄. The highest specific activities toward p-cresol and catechol were found in the 0.41 to 0.46 and 0.46 to 0.52 saturation (NH₄)₂SO₄ precipitates (Table I). An activity relatively high towards p-cresol was found in the 0.60 to 0.66 saturation fraction; this more soluble material has
not been examined closely, but it is probably analogous to the soluble high cresolase fraction observed by Mallette et al. (23).

Pigment Removal by Lead Subacetate (Step 6)—Most of the brown pigments (probably melanins) adsorbed to proteins were removed by stepwise additions of saturated aqueous lead subacetate at 0°. This operation was delicate because excess lead subacetate removed enzyme as well as pigment; with care, 70% recovery could be achieved. To a solution containing approximately 10 mg of proteins per ml, a volume of reagent equivalent to 1/3 of the initial volume was added slowly with gentle stirring. The subsequent additions of reagent were made a few drops at a time, waiting 10 to 15 minutes after each. The pale brown supernatant (yellow if too much lead subacetate was added) was centrifuged at 16,000 r.p.m. and then exhaustively dialyzed first against water and then 5 mM Na2HPO4. The specific activity of the supernatant either increased or remained the same, depending on the preparation. In some cases, we tried to increase recovery by retreated a solution of the pigmented precipitate, which, in general, had only weak activity, but this procedure should be avoided (see below).

Continuous Flow Electrophoresis (Step 7)—If the order here described (Steps 4, 5, 6, and 7) was followed, a solution containing 5 to 15 mg of proteins per ml gave a fairly narrow enzyme band separated from inactive proteins when subjected to continuous flow electrophoresis. Fig. 1 depicts the pattern obtained with a concentrate from 0.42 to 0.46 saturation ammonium sulfate precipitate from which most of the pigments had been removed by lead subacetate. The enzyme was well located, principally in tubes 15 to 19. Fig. 2 depicts the results obtained with cruder 0.35 to 0.73 saturated (NH4)2SO4 precipitate containing 33.5 mg of protein per ml, specific activity toward catechol (12), 100. Tris-HCl buffer, pH 7.65, ionic strength, 0.02; 20 ma, 340 volts. O——O, absorbancy at 280 mμ; each tube diluted 1:5; Δ——Δ, pigment absorbancy (15); X——X, absorbancy at 475 mμ 20 minutes after addition of 20 μl of diluted eluate to 1 ml of 0.5 mM tyrosine, pH 6.8.

Fig. 3. Chromatography of purified tyrosinase on hydroxylapatite. Upper graph, elution pattern after 120 mg of protein, Preparation M, was applied to a 2.2- X 16-cm column equilibrated with 1 mM phosphate buffer, pH 6.8 (see “Experimental Procedure”). The 0.05 to 0.2 M buffer gradient was begun at tube 62. Lower graph, elution pattern after 180 mg of protein, Preparation J, was applied to a 2.2- X 17.5-cm column. The 0.05 to 0.2 M buffer gradient was begun at tube 80. In both graphs, 4.2-ml fractions were collected from the column every 30 minutes.

Heterogeneous; they could not be successfully resolved by this technique.

Hydroxylapatite Chromatography (Step 8)—The pattern of elution of protein fractions from hydroxylapatite columns is depicted in Fig. 3, and properties of the fractions are given in Tables I and II. The tube contents corresponding to each peak were collected, excluding the very diluted portions, the overlapping portions, and portions of suspected heterogeneity, e.g. tubes 75 to 79 in the β-peak of Preparation M (Fig. 3). The α-peak of Preparation J was clearly heterogeneous, but the second section
Molecular ratios of 400, 266, and 133 to 1. Ultracentrifugal analysis of the mixtures (see "Experimental Procedure") showed the appearance of a new, light component (Fig. 6) which comprised 33% of the total protein in the case of the highest ratio, but no enzymic activity was lost. The $s_{20,W}$ values for the enzyme control, and the 400, 266, and 133 molecular ratios were, respectively, (heavier component) 6.61, 6.50, 6.39, and 6.51; and (lighter component) 2.57, 2.49, and 2.33, in the corresponding runs. This experiment indicates that $\beta$-tyrosinase dissociated into subunits in the presence of sodium dodecyl sulfate, although the possibility of unfolding rather than dissociation cannot be

Specific Activities of $\alpha$, $\beta$, $\gamma$, and $\delta$-Tyrosinases—These activities are listed in Table I (Preparation J) and Table II (Preparations F and M). The results are comparable. The $\alpha$ fraction, as observed by Smith and Krueger (8), is able to oxidize catechol but is rapidly inactivated during the reaction. It is, however, very active towards 3,4-dihydroxyphenylalanine and p-cresol. The $\beta$-tyrosinase had high cresolase and high catecholase activities. The $\gamma$-tyrosinase has the highest catecholase and lowest cresolase activities. $\delta$-Tyrosinase, like the $\beta$-tyrosinase, possesses both activities although at lower levels of specific activity. Thus, $\alpha$-tyrosinase can be considered "high-cresolase"; $\gamma$-tyrosinase, "high-catecholase"; and $\beta$- and $\delta$-tyrosinases, mixed catecholase-cresolases. These ratios of phenol $\alpha$-hydroxylating and $\alpha$-diphenol dehydrogenating activities do not extend to other sets of substrates. The activity of $\alpha$-tyrosinase ("high-catecholase") towards 3,4-dihydroxyphenylalanine is greater than that of $\gamma$-tyrosinase ("high-catecholase"), and one cannot generalize concerning the ratio of activities.

None of the four tyrosinases possessed lactase activity when hydroquinone was used as substrate.

Enzyme Stability—We did not systematically study enzyme stability, but decreased activity was always observed after prolonged storage, particularly toward p-cresol. Purified enzymes were divided into small portions and frozen below $-20^\circ$; as a rule, they were less stable than partially purified preparations.

Homogeneity of $\alpha$, $\beta$, $\gamma$, and $\delta$-Tyrosinases—The $\beta$- and $\gamma$-tyrosinases (Preparations F and J) moved as single components during ultracentrifugation (Fig. 4). Several determinations of $s_{20,W}$ extrapolated to zero concentration gave a value of 7.25 for both. $\delta$-Tyrosinase (Preparation M) was essentially homogeneous (Fig. 4); it was not studied at several concentrations, but the once-determined sedimentation coefficient agreed with that of the others. $\alpha$-Tyrosinase, although obtained from well defined chromatographic peaks (Preparations F and M), was heterogeneous by ultracentrifugation (Fig. 4). The heavier component would probably have an $s_{20,W}$ slightly lower than the values found for the $\beta$- and $\gamma$-tyrosinases; a substantial amount of a light component was present in all three preparations of $\alpha$-tyrosinase, although in different proportions. A lighter component was also found in the $\delta$-tyrosinase of Preparation J.

$\beta$- and $\gamma$-Tyrosinases were essentially homogeneous by analytical electrophoresis at pH 6.8 (Fig. 5), although there was an indication of some faster migrating material in $\gamma$-tyrosinase. An average of two determinations of electrophoretic mobilities gave, for $\beta$-tyrosinase, $-6.07 \times 10^{-5}$ (descending) and $-6.40 \times 10^{-5}$ (ascending); for $\gamma$-tyrosinase, the values were $-6.01 \times 10^{-5}$ (descending) and $-6.40 \times 10^{-5}$ (ascending).

Molecular Weights—The molecular weights of $\beta$- and $\gamma$-tyrosinases (Preparation J) found by sedimentation equilibrium (see "Experimental Procedure") were 118,600 and 119,500 for $\beta$, and 118,500 for $\gamma$. The precision was $\pm 10\%$ (22).

Dissociation of $\beta$-Tyrosinase in Presence of Sodium Dodecyl Sulfate—The molecular weight of $\beta$-tyrosinase (Preparation J) was taken as 119,000. Sodium dodecyl sulfate was added in molecular ratios of 400, 266, and 133 to 1. Ultracentrifugal

In 5 mM Na$_2$HP0$_4$; rotor speed, 45,000 r.p.m. $\alpha$ fraction (Preparation F): 3.50 mg of protein per ml; average temperature $= 27.4^\circ$. Photographs were taken at 22, 54, and 85 minutes. $s_{20,W}$ of the heavier component $= 6.41$; of the lighter component, $= 1.49$. $\beta$ fraction (Preparation F): 4.45 mg of protein per ml, average temperature $= 28^\circ$. Photographs were taken at 21, 53, and 85 minutes. $s_{20,W} = 6.03$. $\gamma$ fraction (Preparation F): 3.70 mg of protein per ml, average temperature $= 26.8^\circ$. Photographs were taken at 20, 52, and 84 minutes. $s_{20,W} = 6.24$. $\delta$ fraction (Preparation M): 3.90 mg of protein per ml, average temperature $= 27^\circ$. Photographs were taken at 26, 58, and 74 minutes. $s_{20,W} = 6.20$.  
excluded. A value of 34,500 was once observed for the molecular weight of mushroom tyrosinase (3); the $s_{20,w}$ values now observed for the “subunit” would be consistent with this value. A subunit containing one atom of copper per molecule ($\beta$-tyrosinase contains 0.19% copper) would have a minimal molecular weight of approximately 30,000.

Amino Acid Analysis of $\beta$, $\gamma$, and $\delta$-Tyrosinases—Table III presents the amino acid composition of $\beta$, $\gamma$, and $\delta$-tyrosinases derived from a 20-hour acid hydrolysis. Threonine, serine, proline, cystine, and tyrosine may have undergone some decomposition, and tryptophan is known to be lost under our conditions. More complete analyses will be reported in another paper. However, Table III indicates that a striking likeness exists among the three tyrosinases. The total recovery does not include tryptophan (approximately 6% by the method of Goodwin and Morton (21) in $\beta$- and $\gamma$-tyrosinases, Preparation J) nor ammonia. Tyrosine estimated spectrophotometrically was found to be approximately 30% higher than the Stein and Moore analysis value. Several small, unknown components were also detected during the chromatographic analysis.

Absorption Spectrum—The four tyrosinases were pale yellow in solution and showed very similar absorption spectra, a maximum at 280 to 281 m$\mu$, a shoulder at 290 m$\mu$, and a low, broad shoulder between 320 and 330 m$\mu$ (Fig. 7) with $A_{280} = 2.45$ to 2.65 (1 mg per ml, 1 cm) for the homogeneous $\beta$, $\gamma$, and $\delta$-tyrosinases. The ratio of the absorbancies at 330 m$\mu$ and 280 m$\mu$ were variable even for purified enzymes. They ranged from 0.11 to 0.06, and tended to increase during prolonged storage with the concomitant development of a pale brown color. The mechanism of this process is unknown.

Total Copper Content—The copper contents of the purified tyrosinases are given in Table IV. The values were variable, even for the same type of tyrosinase obtained in different preparations. If one assumes a molecular weight of 119,000, four atoms of copper per molecule corresponds to a copper content of 0.21%, the closest agreement, at least for $\beta$- and $\gamma$-tyrosinases. In spite of precautions to avoid contamination from water, reagents, and glassware, the presence of extraneous copper cannot be ruled out.

Cuprous Copper Content—In determining cuprous copper, the copper is usually released from enzyme by acid. If it is initially in the cuprous state, it may either autoxidize or be bound to a cuprous-specific reagent such as biquinoline, forming a complex which is estimated colorimetrically. If initially in the cupric state, the copper may be reduced by groups on the protein. These circumstances make cuprous copper determination ambiguous. Our results with $\gamma$-tyrosinase (Preparation J) are shown in Fig. 8. Similar results were obtained with the $\alpha$, $\beta$, and $\delta$ fractions of the same preparation. In the absence of added reducing agent, the development of the cuprous-biquinoline color was slow and no well defined plateau was reached, in contrast to the results of Kertesz (10). Ascorbic acid added any time
after the start of the reaction immediately increased the cuprous-biquinoline concentration to roughly the same value, but p-chloromercurisulfonic acid had no effect on the course of the appearance of cuprous-biquinoline. It is clear that cuprous-biquinoline forms slowly after the decomposition of tyrosinase.

### TABLE III

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amino acid residue&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amino acid residue&lt;sup&gt;β&lt;/sup&gt;</th>
<th>Amino acid residue&lt;sup&gt;γ&lt;/sup&gt;</th>
<th>Amino acid residue&lt;sup&gt;δ&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>β</td>
<td>γ</td>
<td>ε</td>
<td>α</td>
</tr>
<tr>
<td></td>
<td>g/100 g protein</td>
<td>moles/mole enzyme</td>
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<tr>
<td>Lysine</td>
<td>4.20</td>
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<td>Histidine</td>
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<td>Arginine</td>
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<td>Aspartic acid</td>
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<td>11.22</td>
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<tr>
<td>Threonine</td>
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<td>Serine</td>
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<td>Glutamic acid</td>
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<td>4.81</td>
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<td>Isoleucine</td>
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<td>Tyrosine</td>
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<td>Phenylalanine</td>
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<tr>
<td>Total</td>
<td>81.00</td>
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<td>865</td>
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</table>

<sup>a</sup> β- and γ-Tyrosinases from Preparation F, δ-tyrosinase from Preparation M.

<sup>b</sup> Biuret determination.

<sup>c</sup> Assumed molecular weight, 119,000.

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### FIG. 7. Absorption spectrum of β-tyrosinase, 0.417 mg per ml in 5 mM Na₂HPO₄.

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### FIG. 8. Time course of the reaction between tyrosinase copper and 2,2'-biquinoline. V—V; 0.1 ml containing 0.65 mg of γ-tyrosinase (Preparation J, 0.29% total Cu) was mixed with 0.2 ml of 0.05%, (weight per volume) p-chloromercuriphenylsulfonic acid, and finally with 0.7 ml of 0.1%, (weight per volume) 2,2'-biquinoline in glacial acetic acid; after 202 minutes, excess ascorbic acid was added. O—O, the same reaction system without p-chloromercuriphenylsulfonic acid; ascorbic acid was added after 35 minutes. □—□, the same reaction system as the last, but ascorbic acid was added 2 minutes after zero time. The dashed line on the left margin (Total Copper) indicates the absorbancy corresponding to the total enzymic copper.

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### FIG. 9. Absorption spectrum of 2,2'-biquinoline, 2.0 mg per ml in 5 mM Na₂HPO₄.

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### TABLE IV

<table>
<thead>
<tr>
<th>Total copper and copper valence distribution in purified tyrosinases</th>
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<tbody>
<tr>
<td>Preparation</td>
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<tr>
<td>-------------</td>
</tr>
<tr>
<td>α, J</td>
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<tr>
<td>α, M</td>
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<td>δ, J</td>
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<td>γ, F</td>
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<td>δ, M</td>
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</table>

<sup>a</sup> Determination according to Stark and Dawson (16); see "Experimental Procedure."

<sup>b</sup> ESR analysis; see "Experimental Procedure."

<sup>c</sup> Biuret determination.
the copper was again converted entirely to cupric copper. The functional significance of cupric copper in tyrosinase is now under investigation.

The electron spin resonance spectra of all tyrosinases were typical of amino acid chelates of cupric copper (24), with hyperfine splitting of approximately 180 gauss and some indication of two bands in the $g = 2.0$ region, due perhaps either to more than one type of cupric copper binding, or to the presence of free radical in addition to cupric copper.

**DISCUSSION**

That mushroom tyrosinase exists in multiple forms, indicated by Smith and Krueger (8), has now been confirmed by the isolation of purified enzymes. Smith and Krueger chromatographed on hydroxylapatite a crude concentrate obtained by extraction with 0.1 M NaCl solution and precipitation with ammonium sulfate at 0.7 saturation. They found five active components, while we observed only four. The difference may arise from the presence of a very soluble tyrosinase in the 0.66 to 0.70 saturated fraction which we set aside and have not yet examined. Otherwise, the relative specificities of our four tyrosinases agree with those found by Smith and Krueger in their first three and fifth peaks. Our absolute activities correspond more closely to those of Mallette and Dawson (2) than those of Smith and Krueger for reasons as yet unknown. Perhaps identical assay techniques would yield more consistent specific activities in different laboratories.

Multiplicity of tyrosinases has now been demonstrated at several levels of the phylogenetic scale (25-33). The identical or almost identical sedimentation constants and electrophoretic mobilities of the mushroom tyrosinases may explain the failure of other investigators who did not use hydroxylapatite chromatography to demonstrate heterogeneity of their apparently pure preparations. In one case (Neurospora), the molecular weights of the multiple enzymes have been shown to vary from 34,000 to over 100,000 (31), the lightest enzyme presumably containing only one copper atom per molecule, an important feature from functional significance of cupric copper in tyrosinase is now under investigation.

**SUMMARY**

Multiple forms of mushroom tyrosinase have been obtained in homogeneous state by a process involving preparative electrophoresis and chromatography on hydroxylapatite. Four enzymes, $\alpha$, $\beta$, $\gamma$, and $\delta$-tyrosinase, were obtained, the last three essentially pure. Although these enzymes possessed partially different activities towards mono- and o-diphenols, the three homogeneous ones had very similar amino acid composition. Both cuprous and cupric copper were present in each enzyme. Apparent dissociation into subunits with retention of activity occurs in the presence of sodium dodecyl sulfate.

**ACKNOWLEDGMENTS**

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**REFERENCES**

The Multiple Forms of Mushroom Tyrosinase: PURIFICATION AND MOLECULAR PROPERTIES OF THE ENZYMES
Simone Bouchilloux, P. McMahill and H. S. Mason


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