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Characterization of Structural Properties for Morphological Differentiation of Melanosomes: I. Purification of Tyrosinase by Tyrosine Affinity Chromatography and its Characterization in B16 and Harding Passey Melanomas

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To characterize how structural properties are related to the morphological differentiation of melanosomes, two forms of melanosomes, commonly seen in mammals, were isolated from B16 and Harding Passey (HP) mouse melanomas. From these morphologically different melanosomes, tyrosinase was solubilized by BRIJ-35 and purified by affinity chromatographies substituted with tyrosinase substrates. We found that tyrosine ethyl ester (TEE) and dopa are effective and specific in retaining tyrosinase as an affinity media and that enzyme retrieval with approximately 100% recovery is possible by a substrate, TEE, or a competitive inhibitor, N-acetyl L-tyrosine. The purified tyrosinase of B16 and HP melanosomes possessed a common antigenic site and revealed little difference in size and the Mikaelis constant for dopa utilization. It is likely that tyrosinase is involved in melanosome morphogenesis only through melanization, and that it is not directly related to the architecture, i.e., lamellar and granular patterns, of the inner matrix.

Since melanosomes reveal a unique morphology, melanocytes and their transformed melanoma cells have been widely used in experimental analysis of cellular differentiation. The melanosomes in mammals are morphologically grouped into 2 forms; (a) ellipsoidal in the outer shape and lamellar or filamentous in the inner matrix and (b) spherical in the outer shape and granular inside. In our previous studies with histochemistry and electron microscopy, we have shown that (a) ellipsoidal-lamellar melanosomes are commonly seen during eumelanogenesis whereas spherical-granular melanosomes are seen during pheomelanogenesis, (b) both ellipsoidal-lamellar and spherical-granular melanosomes originate from a common organelle, (c) the synthesis of these 2 morphologically different melanosomes is interchangeable during a certain stage of hair growth as well as after exposure to UV light, and (d) the switch from one type of melanosomes to the other is controlled by the vesiculoglobular bodies [1-4].

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Abbreviations:
BSA: bovine serum albumin
CBB: Coomassie brilliant blue
dopa: dihydroxyphenyl-alanine
HOA: hydroxylapatite
HP: Harding Passey
mol wt: molecular weight
PAGE: polyacrylamide gel electrophoresis
SDGU: sucrose density gradient ultracentrifugation
SDS: sodium dodecyl sulfate
TEE: tyrosine ethyl ester

Among the melanosomal constituents, tyrosinase and matrix proteins may be the key units controlling the morphogenesis of melanosomes, inasmuch as melanins are assembled to or associated to structural matrix proteins to form "melanoprotein" in the presence of tyrosinase [5-9], though phospholipids may also be involved in some part of the assembly [10-12]. However, biochemical characterization of the melanosomal constituents in terms of melanosome morphogenesis has been limited, partly because of difficulties in solubilizing the matrix proteins and preparing a large quantity of tyrosinase as a native form. In addition, characterization of tyrosinase in animals has been primarily carried out on nonmelanosomal cytosol or chemically-cleaved forms, and has been based on one type of melanosomes [13-16].

The purpose of this study is (a) to introduce a new technique for purification of tyrosinase by affinity chromatography and (b) to compare the mol wt, antigenicity and kinetics of native tyrosinase after partial purification between the melanosomes of ellipsoidal-lamellar and spherical-granular forms, in the hope of better understanding how and to what extent the differences in biochemical properties can be attributed to the elucidation of melanosome morphogenesis.

MATERIALS AND METHODS

Source of Melanosomes

B16 and Harding Paasey (HP) mouse melanomas were serially transplanted subcutaneously into C57BL/6 and BALB/c mice (5- to 6-weeks old), respectively. For the present study, 20-30 mice of each strain were prepared every week over a 4-yr period. Tumors of B16 melanoma were removed at 3 weeks and those of HP melanoma at 8 weeks after transplantation, that is, the time when the tumor reached maximum growth, but was yet nonnecrotic. The excised tumor was immediately frozen in liquid nitrogen and stored at −80°C.

Purification of Melanosomes and Solubilization of Melanosomal Tyrosinase

All purification procedures were carried out in the cold. To avoid proteolytic digestion, 2 mM phenylmethyl sulfonyl fluoride (PMSF), prepared in isopropylalcohol, was included in the buffer. The tumors, usually 20 gm, were minced by scissors, homogenized in phosphate buffer (1 mM, pH 6.8, 2 ml/gm of tumor) with a loosely fitted glass-Teflon homogenizer, filtered through gauze, layered on an equal volume of 0.25 M sucrose and centrifuged (400 g, 3 min). Melanosomal suspension above the sucrose layer was collected. This step was repeated until large cell fragments were sedimented and the suspension of melanosomes became homogenous under light microscopy. The suspension was then centrifuged (105,000 g, 30 min) and the supernatant was stored at −80°C for an enzyme source of nonmelanosomal, cytosol tyrosinase. The melanosome pellet was resuspended in sodium phosphate buffer (1 mM, pH 6.8) containing 2 mM PMSF and were applied to discontinuous sucrose density gradient ultracentrifugation (SDGU) (105,000 g, 2 hr; Hitachi RPS-25 swing rotor). The sucrose concentrations were 2.0 M, 1.8 M, 1.6 M, 1.5 M, 1.4 M, 1.3 M and 1.2 M. The melanosomal fraction was obtained at the bottom of 2.0 M sucrose.

Purified melanosomes were exposed to 0.1% BRIJ-35 (polyoxyethylene (23 mol) lauryl ether: Atlas Chemical Industries, Inc., Delaware, U.S.A.) in sodium phosphate buffer (1 mM, pH 6.8). BRIJ treatment was carried out with a Teflon-glass homogenizer (1,000 rpm, 3 min in

the cold). The homogenate was centrifuged and melanosomal tyrosinase was obtained in the supernatant.

Polyacrylamide Gel Electrophoresis (PAGE)

Disc-PAGE was done in either slab or cylindrical gels with or without SDS. Standard PAGE (7.5%) without SDS was performed by the method of Davis [17] with a stacking gel omitted. SDS-PAGE with 7.5% and 10% gels was carried out in 2 different buffer systems described by Weber and Osborn [18], and Laemmli and Favre [19], respectively. The samples were dialyzed against the sample buffer containing 1% SDS. Usually 150 μg protein of samples was applied to each tube or slot for fractionation of protein-subunits. Gels of a protein pattern were stained with 0.1% Coomassie brilliant blue (CBB) R-250 overnight and were destained with methanol-acetic acid solutions. Gels of an enzymatic pattern were washed with cold sodium phosphate buffer (0.4 M and 0.2 M, pH 6.8, each with 3 changes) and were incubated in 0.1% L-3,4 dihydroxyphenyl-alanine (dopa) in sodium phosphate buffer (0.1 M, pH 6.8) at 37°C for 30 min.

Affinity Chromatography for Isolation of Native Tyrosinase

The native form of tyrosinase was partially purified from the BRIJ-solubilized material by ionic exchange chromatographies of DEAE cellulose (DE 52, Whatman) and hydroxylapatite (HOA), and by affinity chromatographies of tyrosinase substrates.

Affinity media were prepared with tyrosinase-substrate, i.e., L-tyrosine ethyl ester (TEE) or L-dopa, which was substituted to Sepharose 4B with cyanogen bromide activation [20]. Usually 15 gm of the activated Sepharose cake was stirred with 50 ml of 1 M KH_2PO_4 (pH 7.0) containing 1.22 g of L-TEE or L-dopa in the cold overnight. The amount of TEE or dopa bound to Sepharose 4B was determined spectrophotometrically by the following formula;

$$\left[1 - \frac{D(B + D)}{AB}\right] \times \frac{E}{C} \times \frac{10^6}{F} = \mu \text{ moles bound/mg of Sepharose.}$$

Where A is OD_{280} of TEE or dopa solution before addition of Sepharose; B is the ml of TEE or dopa solution before addition of Sepharose; C is the gm of Sepharose added to the TEE or dopa solution; D is OD_{280} of the supernatant of the TEE or dopa-sepharose mixture after reaction overnight; E is the gm of TEE or dopa before the addition of Sepharose; F is the moles of TEE or dopa added. By this procedure, about 10 μmoles of TEE and 15 μmoles of dopa per gm of Sepharose was coupled.

Enzyme Units and Protein Concentration

Tyrosinase (dopa-oxidase) activity, expressed in units of enzyme, was determined spectrophotometrically by the amount of enzyme catalyzing the oxidation of 1 μmole of L-dopa to "dopa-chrome" in 1 min at 37°C. The absorption maximum of dopa-chrome was measured at 475 nm with a molecular extinction coefficient of 3,600 [21]. Protein content was measured by the Folin-Lowry method [22] after boiling the melanosomal sample in 1 N NaOH (30 min, 100°C) and subtracting the absorbancy of melanin from the experimental sample. Bovine serum albumin (BSA) was used as a standard protein. Protein concentration was carried out by lyophilization after dialysis against sodium phosphate buffer (1 mM, pH 6.8), by molecular concentration, or by a passage through a small DEAE cellulose column (DE52, Whatman). Molecular filtration was carried out in an ultrafiltration cell with a UM-10 membrane (Amicon Corporation). Enzyme retrieval of tyrosinase from a DE52 column was made by eluting with 0.25 M NaCl in sodium phosphate buffer (1 mM, pH 6.8).

Enzyme Assay

The Km values of L-dopa utilized by the melanosomal tyrosinases of B16 and HP were compared by the method of Pomerantz [23]. The rate in oxidation of L-dopa was determined by measuring absorbancy at 475 nm, an absorbancy maximum of dopa-chrome. The kinetics were carried out in 1 ml of the reaction-mixture containing L-dopa (0.06 to 3 μmoles), tyrosinase (4 units of dopa-oxidase activity) and sodium phosphate (35 μmoles, pH 6.8) at 37°C for 20 min. Under these conditions, the rate of melanin synthesis (dopa-chrome) was linear and proportional to the amount of enzyme added.

Preparation of Antibody

Antibody against nonmelanosomal, cytosol tyrosinase was produced in rabbits by subcutaneous injections of approximately 100 μg per rabbit of partially purified tyrosinase. The tyrosinase was isolated by electrophoretic fractionation of the DEAE (DE52) and HOA purified materials (peak II of the HOA column eluted at 10 mM of phosphate buffer, pH 6.8) and was emulsified with Freund's complete adjuvant. A second immunization (1 week later) was carried out with incomplete adjuvant. The rabbits were rested for 3 weeks and then intravenous booster injection of tyrosinase (approximately 100 μg protein) was repeated at 1-week intervals. Antisera were titered by Ouchterlony double immunodiffusion and immunoelectrophoresis in agar.

Molecular Weight Determination by SDS-PAGE

A mixture of the marker proteins, approximately 5–10 μg each, was simultaneously electrophoresed in duplicate on each side of the gels, and their relative mobilities (Rx) were plotted as a function of the logarithm of respective mol wt. The mol wt of the tyrosinases of B16 and HP was compared by dopa-incubation of a slab SDS-PAGE, using Apo-ferritin (480,000), RNA polymerase (β':165,000, β:155,000, α: 39,000), phosphorylase A (94,000), BSA (68,000), trypsin inhibitor (21,500) and ribonuclease A (13,700). The sample was prepared by dialyzing against sample buffer with 0.1% SDS (overnight, in the cold).

Molecular Weight Determination by Gel Filtration

A column (0.9 × 86.5 cm) of Sephadex G-150 equilibrated in phosphate buffer (50 mM, pH 6.8) was prepared. A fraction of 1.5 ml was collected at a downward flow rate of 3 ml/hr in the cold. The column was calibrated by chromatographing the following proteins: phosphorylase A, BSA, ovalbumin and cytochrome C (13,000). Blue dextran was used to determine the void volume of the column.

RESULTS

Solubilization of Tyrosinase from Melanosomes by BRIJ-35

The buffer system used for melanosome purification was hypotonic, inasmuch as the melanosomes were stable in hyper- and hypotonic media whereas membranous organelle other than melanosomes were vacuolated by these media, thus enabling us to better purify the melanosomes by SDGU. After SDGU purification, about 150 mg and 200 mg (dry weight) of melanosomes were isolated from 1 gm of B16 and HP melanomas, respectively. Under electron microscopy, melanosomes from B16 melanoma were ellipsoidal in the outer shape and contained melanized lamellae and filaments. Melanosomes from HP melanoma were spherical in the outer shape and contained melanized granular materials (Fig 1-*a, b*). Lyophilized materials of the isolated melanosomes were brown-black in B16 and reddish brown in HP.

Our previous study [24] showed that BRIJ-35 was the most potent agent for releasing tyrosinase as an active and native form. In the present study, the BRIJ treatment solubilized about 5–6% of the total proteins of the melanosomes in B16 and HP.

Separation of Melanized and Nonmelanized Tyrosinases

All the column chromatographies were carried out by sodium phosphate buffer [23]. The DEAE cellulose column was eluted by NaCl, instead of KCl as reported by Brown and Ward [25], and Nishioka [16].

BRIJ-solubilized materials were separated into 2 tyrosinase fractions on a Sephadex G-200 column, i.e., the 1st eluted at the area corresponding to a void volume and the 2nd at the area of the mol wt between 94,000 (phosphorylase A) and 68,000 daltons (BSA), respectively (data not shown). When applied to a disc-PAGE (7.5%), the aliquot of the 1st fraction showed a band with the brown color of melanin (Rx:0.13) whereas the 2nd fraction was colorless (Rx:0.56) (data not shown).

Ionic-exchange chromatographies on DEAE cellulose and HOA completely separated the least or nonmelanized tyrosinase from that which was heavily melanized. When BRIJ-35 solubilized materials were applied to a DE52 column with a linear gradient of NaCl, the tyrosinase fraction was recovered at 0.25 M of NaCl (Fig 2-*a*). The pooled material of this tyrosinase fraction migrated to the area corresponding to that of the second peak of tyrosinase on Sepharose G-200. When the tyrosinase fraction from a DE52 column was subsequently applied to a HOA column with a linear phosphate gradient, further purification of tyrosinase was achieved (Fig 2-*b*).

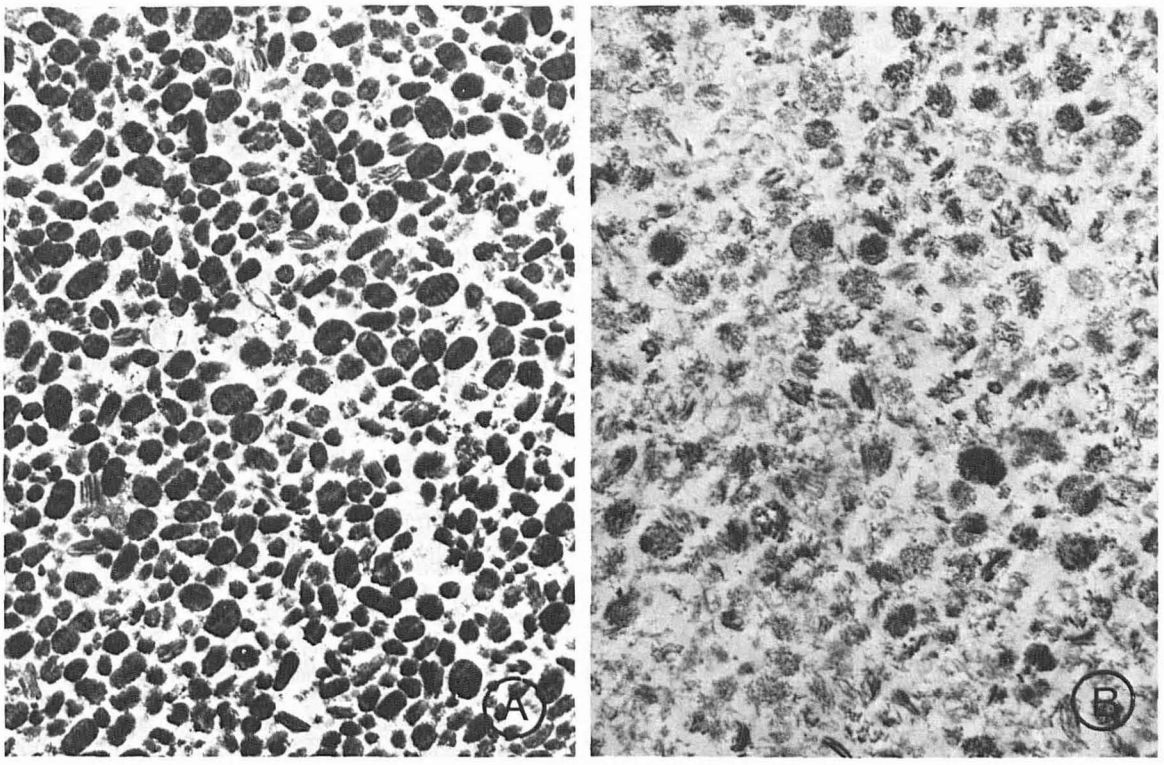

FIG 1. Ultrastructure of melanosomes after purification by SDGU. *A*, B16 melanosomes (× 8,500); *B*, HP melanosomes (× 8,900).

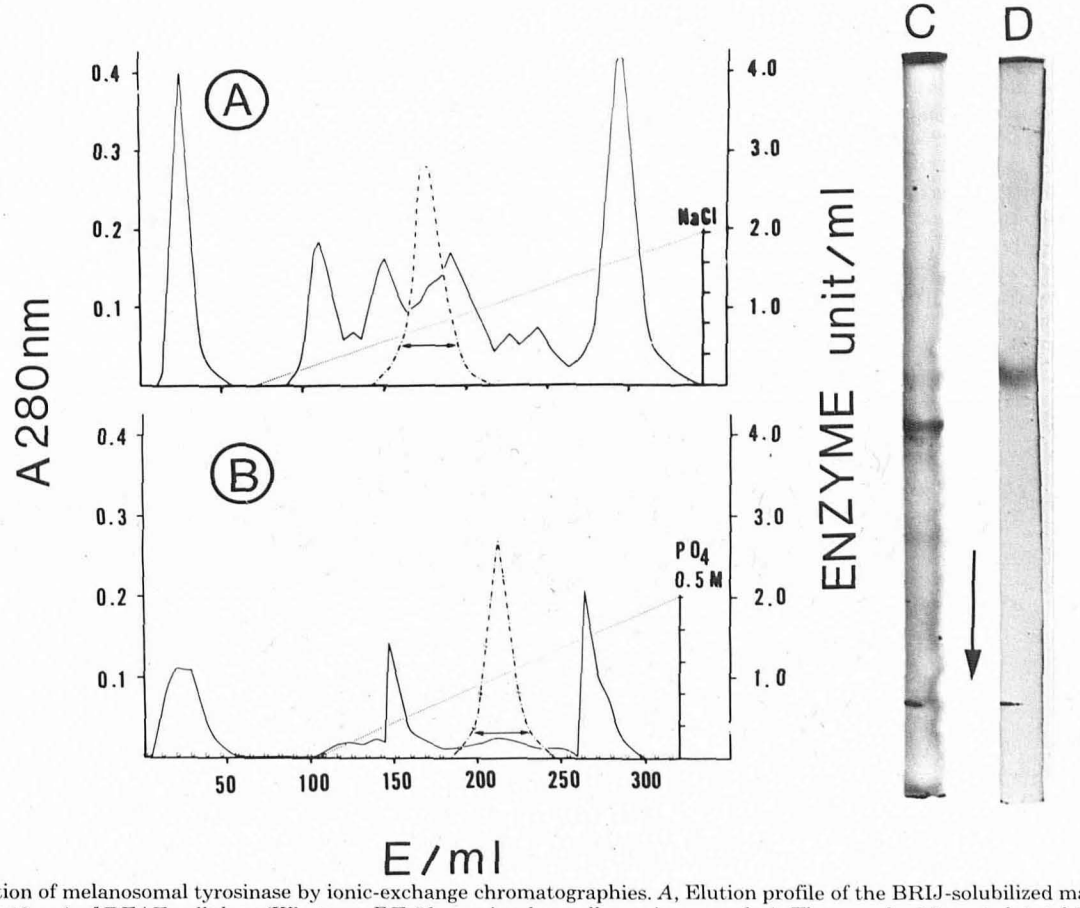

FIG 2. Isolation of melanosomal tyrosinase by ionic-exchange chromatographies. *A*, Elution profile of the BRIJ-solubilized materials of HP on a column (1.9 × 10 cm) of DEAE-cellulose (Whatman DE-52, previously swollen, microgranular). The sample, 5.9 mg/ml, total 50 ml, was eluted with a linear gradient of NaCl (0 to 0.5 M) in phosphate (1 mM, pH 6.8). Fractions, 3 ml each, were collected at a rate of 6 fractions per hr. *B*, Elution profile of the melanosomal tyrosinase of HP on a HOA column. The materials, 45 ml, were the pool of the dopa-oxidase fraction from a DE52-column (Fig 2-*A*). The HOA column (1.9 × 10 cm) was previously equilibrated with phosphate buffer, 1 mM, pH 6.8. After sample loading, the column was flushed with the starting buffer and eluted with a linear gradient of phosphate (1 mM to 0.5 M). Fractions, 2 ml each, were collected at a rate of 6 fractions per hr. The *insert* is the disc-PAGE (7.5%) showing the purity of melanosomal tyrosinase after HOA column. The left gel is stained with CBB (*C*) and the right with dopa-incubation (*D*).

Purification of Tyrosinase by Affinity Chromatography Using the Tyrosinase-Substrates

The purification of tyrosinase was further achieved by affinity chromatography of either L-TEE or L-dopa (Fig 3). Because of its limited solubility, tyrosine was not used (maximum solubility:2.5 mM at 25°C). TEE can act as a substrate for tyrosinase with the same activity as tyrosine [13] and can be easily solubilized (up to 8 mM at 25°C). Figure 3 is an example of affinity chromatography in which TEE was used to immobilize tyrosinase. During the loading and flushing phases in the presence of 0.1 M NaCl, protein was being eluted from the column, but it was completely devoid of tyrosinase activity. When dopa was used as affinity resin, the top of the column became dark-brown during sample loading and flushing phases. The darkened column could be regenerated by washing the column with 5 mM ascorbic acid, which reduced dopa-quinone to dopa. During sample loading and flushing phases, tyrosinase immobilized 100% of enzyme activity to the TEE column and 85% to the dopa column.

When all the protein not having an affinity for TEE or dopa had been removed, the tyrosinase was then displaced by flushing the competitive inhibitor (2% N-acetyl L-tyrosine) or the substrate (2% TEE), or by flushing with 6 M urea, which weakened the tyrosine-tyrosinase complex. When N-acetyl L-tyrosine was used to elute tyrosinase complexed to the affinity resin, the protein displaced was masked by the high optical density of the eluting material and could not be detected until N-acetyl L-tyrosine was removed by an extensive dialysis against phosphate buffer (1 mM, pH 6.8). In contrast, when TEE was used, enzyme was easily retrieved with a small DE52 column where tyrosinase was bound at low ionic strength, but the positively charged TEE passed through. When urea was used to retrieve the tyrosinase, a small protein peak which contained most of the tyrosinase activity was eluted at approximately 3 M urea. An *insert* of Figure 3 shows the SDS-PAGE of protein (C:CBB) and tyrosinase activity (D:dopa staining) after purification by TEE affinity chromatography. Overall yield in the final step based on recovery of the enzyme units present was about 50% and the specific activity of the purified tyrosinase increased 2,000 times over the original melanosomes (Table). The tyrosinases of B16 and HP showed similar elution profiles on DE52, HOA and TEE affinity columns.

Summary of purification of tyrosinase from melanosomes

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg)	Recovery (%)
Melanosome fraction[a]	50	102.0	0.003	100
BRIJ supernatant	67	6.8	0.031	91
DEAE-cellulose	50	0.36	0.49	58[b]
Hydroxylapatite	50	0.12	1.25	49
Tyrosine affinity	31	0.04	5.88	48

[a] Melanosomal fraction isolated from 25 gm of Harding Passey tumor (wet weight). B16 melanosomes also revealed a simular result for purification.

[b] Rest of tyrosinase was highly melanized and not eluted from the column.

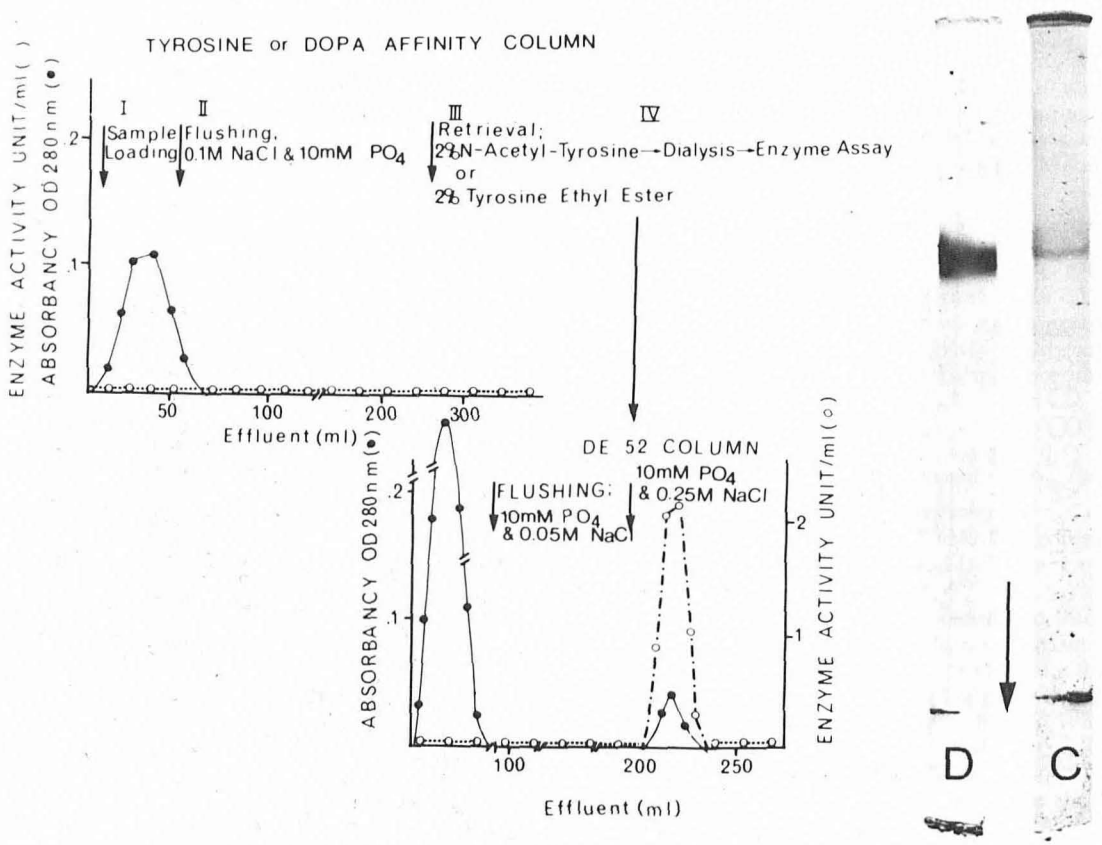

FIG 3. Elution profile of HP tyrosinase in tyrosine and dopa affinity chromatography. A sample, partially purified by DE52 and HOA columns (Fig 2-*A, B*), was dialyzed against phosphate buffer (10 mM, pH 6.8) in the presence of 0.1 M NaCl and applied to an affinity column, 0.9 × 10 cm. The column was previously equilibrated with the same buffer and NaCl. Tyrosinase was completely absorbed on the column and the protein not having tyrosinase activity was eluted. After flushing with phosphate buffer with NaCl, the tyrosinase fraction was eluted with 2.0% TEE·HCl in phosphate buffer (3 times the volume of column). The eluate was collected, dialyzed against phosphate buffer and then applied to a small column (0.9 × 5 cm) of DE52 (previously equilibrated with the same buffer). During the sample loading and flushing phases, the eluate with a high optical density at 280 nm passed through. Tyrosinase was recovered as a small protein peak with a high enzyme activity by eluting the column with 0.25 M NaCl in phosphate buffer (10 mM, pH 6.8). By this procedure, tyrosinase was recovered as as much as almost 100% of total enzyme activity. The *insert* is of the gels with a SDS-PAGE (10%) of the materials after the TEE column (*C*, CBB-staining and *D*, dopa-staining).

Kinetics, Molecular Weight and Antigenic Site of Tyrosinase

Kinetic constants for dopa, mol wt and antigenic site were compared between the column-chromatographically isolated tyrosinases of HP and B16. When the pH-optimum profile was determined between pH 5.6 and 7.8, the activity was maximum between 6.6 and 7.0. The activity at pH 5.6 was one-half that of the maximum in both HP and B16. Because of the inherently low enzymic activity of B16 tyrosinase [26], comparison of tyrosine kinetics between B16 and HP was not carried out. The Km for dopa was measured as 3.0×10^{-4} M in HP and 5.6×10^{-4} M in B16 by Leinweaver-Burk plots.

The mol wt of HP and B16 tyrosinases was compared by SDS-PAGE and Sephadex gel filtration. On an SDS-PAGE, a linear relationship existed between the relative mobilities and mol wt (a plot of the log form) of the standard proteins, ranging in size from 13,000 (cytochrome C) to 94,000 daltons (phosphorylase A). The tyrosinases of B16 and HP, electrophoresed either each separately or mixed together, migrated to an identical position. The mol wt of tyrosinase was estimated as 73,000 ± 800 daltons for B16 and 72,700 ± 500 daltons for HP (mean ± standard error of 5 experiments). A similar result for mol wt was obtained by calculation with Sephadex G-150, revealing 74,000 ± 1,000 daltons for B16 and 74,000 ± 1,800 daltons for HP tyrosinase.

Figure 4 shows the result of immunoelectrophoresis, demonstrating that melanosomal tyrosinases of B16 and HP possess a common antigenic site with a precipitation line of cross-reactivity to the nonmelanosomal, cytosol tyrosinase of HP. The cross-reactivity of the tyrosinases of B16 and HP was also confirmed by Ouchterlony immunodiffusion.

DISCUSSION

In an early affinity chromatrography attempt, Lerman [27] explored the use of 2 cellulose derivatives in the purification of plant (mushroom) tyrosinase. The functional moieties of the derivatives were a phenol (a substrate) and a substituted benzoic acid (an inhibitor). Later, modifications of Lerman's original technique were reported by Gutteridge and Robb [28], and O'Neil, Graves and Ferguson [29], by whom a benzoic acid derivative of agarose and a dopamine, dihydroxyphenyl derivative, bound to Sepharose were successfully used in purifying mushroom tyrosinase. However, utilization of affinity chromatography for purification of mammalian tyrosinase has been limited. Nishioka [16] recently succeeded in partially purifying melanoma-tyrosinase by concanavalin A affinity chromatography, which is based on the content of sialic acid and is rather nonspecific. In our system, compounds having a specific biological affinity for the tyrosinase to be purified were covalently bound to an insoluble matrix of Sepharose. We found that TEE or dopa, a substrate of tyrosinase, substituted to the side-arm of Sepharose, is effective and specific in retaining tyrosinase. Enzyme retrieval with approximately 100% recovery was possible by eluting the column with a substrate, TEE, or a competitive inhibitor, N-acetyl L-tyrosine. The results were reproducible provided that the preparations were first decolorized by ionic exchange chromatographies of DEAE and HOA. The tyrosinase isolated by TEE affinity chromatography corresponded to the main component identical in relative mobility of protein (CBB) and enzyme (dopa) on the gels, though it might not be pure enough to show a homogeneity.

Characterization of melanosomal tyrosinase by previous studies [14,16] has been primarily based on trypsin-solubilized materials. Our present study showed that native tyrosinases of morphologically different melanosomes solubilized by BRIJ possess a common antigenic site, and reveal little difference in size as well as in the Mikaelis constant for dopa utilization, which was, however, in agreement with previously reported ones [23, 30]. The estimated size was different from that of the trypsin-treated material of human melanoma (66,700 daltons) [16]. Our previous cytochemical study [1] indicated that most of active tyrosinase is present below the outer membrane of melanosomes and that some of it is associated with melanin moieties complexed with the inner matrix. The elution profile of BRIJ-solubilized tyrosinase on the Sephadex column, in which active tyrosinase was discerned into (a) heavily and (b) least melanized fractions, may support this view. The electron microscopic study of Birbeck [31], however, proposed that the inner structure of melanosomes represents a 2 dimensional array of tyrosinase molecules which behave simultaneously as both structural proteins and active enzymes. Our present study suggested that tyrosinase is involved in the morphogenesis of melanosomes only through melanization of melanosomes, and that it is not primarily involved in the architecture, i.e., lamellar and granular patterns, of the inner matrix. For the elucidation of melanosome morphogenesis, it is necessary to study other structural properties such as structural matrix proteins, lipids, and melanins of melanosomes, and the mode of their three-dimensional assembly.

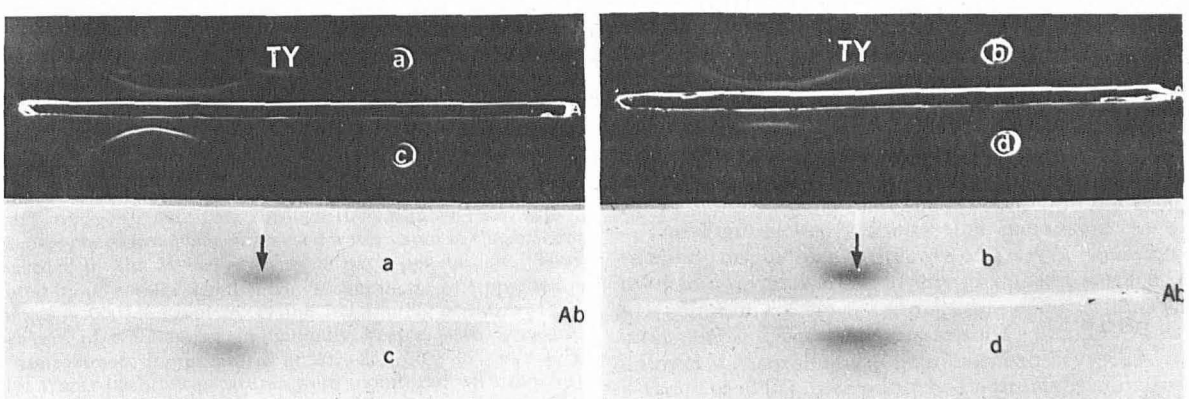

FIG 4. Immunoelectrophoresis showing the cross-reactivity of melanosomal tyrosinase of B16 and HP with the antibody developed against non-melanosomal tyrosinase of HP (peak II of HOA, eluted at 10 mM phosphate buffer). Antigens, partially purified by DE52 and HOA column chromatographies, were placed in the circular wells, electrophoresed and then reacted with antiserum (*Ab*) placed in the center. After the development of the precipitation line (24 hr in a cold room), the gels (*top gels*) were washed with cold phosphate buffer and then incubated in 0.1% dopa-phosphate solution for 30 min at 37°C (*bottom gels*). (*a*) Reaction with melanosomal tyrosinase (10 μg representing 5 μl of 2 mg/ml solution) of HP. (*b*) Reaction with melanosomal tyrosinase of B16. (*c*) Reaction with nonmelanosomal tyro'inase of B16 (peak I of HOA, eluted at 3 mM phosphate buffer). (*d*) Reaction with nonmelanosomal tyrosinase of B16 (peak II of HOA, eluted at 10 mM phosphate buffer). *Arrows* indicate the dopa-positive tyrosinase (*TY*). Both B16 and HP melanosomes possess tyrosinase cross-reacting to the antibody against the nonmelanosomal, cytosol tyrosinase of HOA (peak II of HOA). The antiserum used is the unabsorbed material, in which the antibody against serum albumin is also contained.

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