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PROPERTIES AND STATE OF PARTICULATE TYROSINASE OF XANTHIC GOLDFISH

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SUMMARY

1. The properties, subcellular distribution and status of particulate tyrosinase in xanthic goldfish skin were studied using fractionated materials.

2. Tyrosinase occurred in two molecular forms that exhibited different electrophoretic mobilities in acrylamide-gel electrophoresis.

3. Freshly prepared particulate or pterinosome suspension in an isotonic medium had no measurable tyrosinase activity. The enzyme activity was detected after mechanical or chemical breakage of the structural integrity of the particles.

4. No endogenous tyrosinase inhibitor was found in the particulate fractions.

INTRODUCTION

The presence of tyrosinase in xanthic goldfish skin has been reported, although this variety lacks melanin pigmentation in the integument[1–4]. The enzyme activity is demonstrable *in vitro*, but a histochemical DL-3,4-dihydroxyphenylalanine (DL-DOPA) reaction is negative[5]. As to the intracellular distribution of the enzyme, LOUD AND MISHIMA[6] suggested that tyrosinase is present in the pigment granules within xanthophores. This was confirmed by recent experiments[7,8] in which the occurrence of tyrosinase in the fractionated pigment granules (pterinosomes) was ascertained. It was also found that the enzyme activity in the particulate fraction is enhanced by sonication[9]. This suggests that tyrosinase in pterinosomes is structure-linked and is in latent form *in vivo*. The present study deals with the properties, subcellular distribution and state of tyrosinase in the integument of xanthic goldfish.

MATERIALS AND METHODS

Goldfish of the fan-tailed variety *(Carassius auratus)* weighing 9–15 g were purchased from a hatchery. Fins and skins taken from freshly killed animals were rinsed in chilled 0.24 M sucrose, gently homogenized in a Teflon homogenizer, and filtered through a double-layered gauze and a cell strainer. Preparation of the large granule fraction and further fractionation of subcellular particles using sucrose

density gradient centrifugation were carried out as described before[7]. Three to ten fish were killed for the preparation of the large granule fraction, and fifteen to thirty fish were used for fractionation by density gradient centrifugation. Tyrosinase assay was made manometrically and colorimetrically. Manometric assay was carried out for the study of substrate specificity, and the colorimetric method was used routinely for the determination of enzyme activity.

Manometric assay

The central well of a Warburg manometer vessel contained 0.1 ml of 20% KOH, the main compartment contained 1.0 ml of the enzyme solution and 0.5 ml of 0.1 M phosphate buffer (pH 6.8), and the side arm contained 0.5 ml of substrate. The substrates were 16.7 mM L-tyrosine dissolved in 16.7 mM NaOH, L-tyrosine containing DL-DOPA at a concentration of 1.67 mM, 16.7 mM DL-DOPA, catechol and *p*-cresol dissolved in distilled water. The large granule preparation homogenized in 0.4% sodium deoxycholate in 0.05 M Tris buffer (pH 7.8) was centrifuged at 164 000 × *g* for 60 min, and the supernatant was used as enzyme solution. About 90% of the total tyrosinase activity was recovered in the supernatant fraction. Enzyme activity was measured at 30°.

Colorimetric assay

The standard assay system contained 0.5 ml of 0.04 M DL-DOPA dissolved in distilled water, 0.5 ml of enzyme solution and 1.0 ml of 0.2 M phosphate buffer (pH 6.8). Enzyme solution was prepared from the large granule preparation of materials fractionated by density gradient centrifugation. Sonication of the material, suspended in 0.05 M phosphate buffer (pH 6.8) was carried out for 2.5 min at 20 kcycles/sec in an ice bath. Enzymic digestion with trypsin (Merck), chymotrypsin (Merck) and phospholipase A (Boehringer) was carried out as follows: 1 mg of enzyme dissolved in 1.0 ml of 0.001 M HCl (trypsin and chymotrypsin) or distilled water (phospholipase A) was diluted 10 times, and 0.1 ml of the solution was added to 1.0 ml of the material suspended in 0.05 M Tris buffer (pH 7.5) and allowed to stand for 60 min at 25°. The intensity of the color of dopachrome was measured by a Leitz photometer with a 490-nm filter. 1 tyrosinase unit is defined as the amount that increases the absorbance by 0.001 per 15 min at 25° under the experimental conditions used.

Electrophoresis

Acrylamide-gel electrophoresis was carried out according to the method of DAVIS[10]. Materials were either sonicated or solubilized with deoxycholate. Deoxycholate was removed by gel filtration through a column (9 mm × 90 mm) of Sephadex G-25. An aliquot of 0.15 ml of the material containing 150–300 μg protein was submitted to electrophoresis for 2–3 h at 2° (1.5 mA/tube). After separation, gels were incubated in 0.3% DL-DOPA dissolved in 0.1 M phosphate buffer (pH 6.8) for 1.5–2 h at 37° or 20 h at 2°.

Protein was measured by the method of LOWRY *et al.*[11].

RESULTS

Goldfish enzyme oxidizes DOPA but it does not oxidize cresol or catechol. When

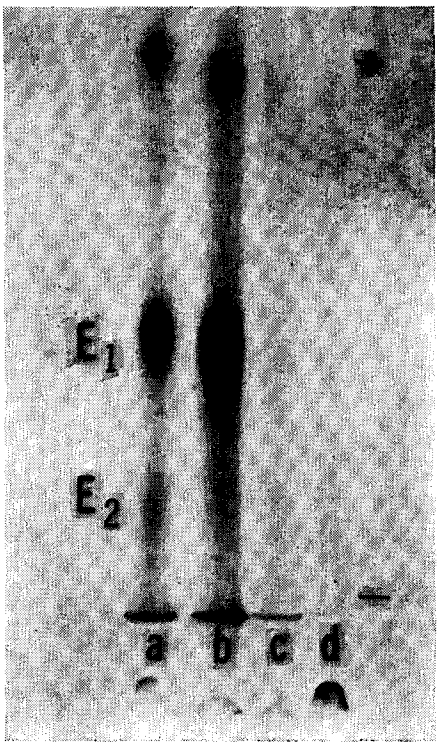

Fig. 1. Electrophoretic patterns of tyrosinase in particulate fractions. a, large granule preparation; b, pterinosome fraction; c, mitochondrial fraction; d, smooth membrane fraction; E_1, fast-migrating component; E_2, slow-migrating component. Each fraction was sonicated before use. Gels were incubated in DOPA for 20 h at 2°.

tyrosine was used as substrate, the activity was measurable only in the presence of a small amount of DOPA as a primer, the K_m for DOPA being 0.25 mM. The enzyme activity was totally inhibited when phenylthiourea, diethyldithiocarbamate or KCN was added to the reaction mixture at a concentration of 1 mM. Enzyme activity increased with temperature with a Q_{10} of about 2 in the range of 2–30°.

The electrophoretic pattern of the enzyme from particles is shown in Fig. 1. Two components of tyrosinase having R_x values of 0.50 and 0.17 were detected in

TABLE I

DISTRIBUTION OF TYROSINASE IN PARTICULATE FRACTIONS

The large granule preparation obtained from the fins of 21 goldfish (18 g) was suspended in 4 ml of 0.24 M sucrose; 3 ml of the suspension was layered on the density tubes. Sonicated materials were used for enzyme assay.

Fraction	*Total activity (units)*	*Specific activity (units/mg)*
Large granule	960	35
Pterinosome	464	82
Mitochondrial	54	14
Smooth membrane	60	11

TABLE II

ACTIVATION OF PARTICULATE TYROSINASE

Expt.	*Treatment*	*Relative activity (units)*
1*	Suspended in 0.24 M sucrose	0
	Sonication	84
2**	Suspended in water	37
	Suspended in 0.05 M Tris buffer	21
	Trypsin	21
	Deoxycholate	45
	Sonication	64
3**	Chymotrypsin	38
	Trypsin	41
	Phospholipase A	66
	Deoxycholate	66
4**	Sonication–standing at 0°, 60 min	52
	Sonication–standing at 25°, 60 min	55
	Sonication–deoxycholate	55
	Sonication–chymotrypsin	55
	Sonication–trypsin	48

* Pterinosome fraction.
** Large granule preparation.

the large granule and pterinosome preparations (Fig. 1, a, b). The rapidly migrating enzyme was very distinct while the slow-moving component was rather diffuse. Mitochondria and smooth membrane fractions had no distinct DOPA-positive band (Fig. 1, c, d). The electrophoretic patterns of the supernatant fraction and total skin homogenate were qualitatively the same as that from the large granule preparation, but the enzyme activity seemed to be low in the supernatant fraction.

Among particulate fractions, tyrosinase was present predominantly in pterinosomes while mitochondria and smooth membrane had low enzyme activities (Table I).

Activation of enzyme in particles by physical and chemical treatments was studied using the large granule and pterinosome fractions (Table II). Unbroken pterinosomes suspended in an isotonic solution showed no appreciable enzyme activity, but sonicated or homogenized material in distilled water had comparatively high enzyme activity. Solubilization with deoxycholate and enzymic digestion with phospholipase A were also effective in activating tyrosinase, while trypsin and chymotrypsin had no effect. No further activation was observed in sonicated material by additional treatment with deoxycholate.

In order to test for the presence of endogenous inhibitors, various dilutions of the enzyme preparation were incubated under various substrate concentrations ranging from 0.1–5 mM in terms of L-DOPA, but the amount of dopachrome formed was exactly proportional to the concentration of the enzyme solution. In addition, when goldfish enzyme in various concentrations was combined with crude potato tyrosinase, the amount of dopachrome formed was proportional to that of the enzymes present in the reaction mixture, indicating that there was no tyrosinase inhibitor in

the goldfish preparation. Since pteridines are present in high quantity in goldfish pterinosomes, an attempt was made to see if pteridines have any inhibitory effect on tyrosinase activity. The large granule preparation solubilized with deoxycholate was submitted to gel filtration through a column (13 mm × 150 mm) of Sephadex G-25 in order to remove endogenous pteridines, and the protein fraction was used as enzyme solution. Isoxanthopterin (Aldrich) or 2-amino-4-hydroxypteridine (K and K.) dissolved in 0.01 M NaOH was added to the reaction mixture at a final concentration of 0.125 mM, but these compounds showed no inhibitory effect on goldfish enzyme. Moreover, no inhibition of tyrosinase activity was observed when endogenous pteridines fractionated by gel filtration were recombined with the protein fraction. Addition of a sulfhydryl blocking agent, iodoacetamide, at a final concentration of 0.01 M did not affect the enzyme activity even at low substrate concentrations.

DISCUSSION

Multiple forms of vertebrate tyrosinase have been reported in mammals[12], amphibia[13] and fish[14,15]. In goldfish, there are at least two electrophoretically different types of tyrosinase in the skin. Unfortunately, we have not yet succeeded in isolating these two components; hence, the significance or possible role of these enzymes remains to be studied.

The structure-linked latency of goldfish tyrosinase is similar to that observed in mammalian melanosomes[16] or that found in mitochondrial or lysosomal enzymes[17,18]. This situation explains why goldfish pterinosomes remain unmelanized *in vivo*, despite the presence of tyrosinase. IDE AND HAMA[8] reported that the presence of tyrosinase in erythrophores is demonstrable histochemically after incubation of the tissues in a saline–tyrosine solution for 3 days in the presence of iodoacetamide. It seems probable that under this incubation condition, pterinosomes suffered physiological denaturation which eventually led to the activation of latent enzyme.

In the present study, endogenous tyrosinase inhibitors within the large granule preparation or pterinosome fraction were not detected. Acceleration or inhibition of *in vitro* melanization by some of the oxidized pteridines has been reported[19,20], but we have not evidence that endogenous pteridines in goldfish pterinosome inhibit the melanization *in vivo*. Goldfish pterinosomes contain sepiapterin in a small amount[7], but it is not known if they contain some other pteridines in reduced form.

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