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Monophenol Oxidase Activity from the Cuticle of Florida Spiny Lobster (*Panulirus argus*)†

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Endogenously activated phenoloxidase (PO) from the cuticle of Florida spiny lobster was purified 37-fold. It had an isoelectric point of 4.76 and an apparent molecular weight of 81 200 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Ion-pair reversed-phase high-performance liquid chromatography demonstrated that PO can catalyze the hydroxylation of L-tyrosine to dihydroxyphenylalanine (mono-PO activity). The optimum pH for hydroxylation was between 6.0 and 6.5, and the PO was most stable from pH 6 to 8. The optimum temperature for reaction was 40 °C, and PO was stable up to 40 °C. The apparent Michaelis–Menten constant (K_m) was 0.97 mM for PO hydroxylation of L-tyrosine.

INTRODUCTION

Crustacean integumental glands, which show cyclical activity with molting, contain a phenol oxidase (PO) believed to be involved in sclerotization (Pryor, 1962). Although PO occupies the central position in sclerotization of the arthropod cuticle (Brunet, 1980), information on this enzyme in crustacean cuticle is sparse. Few attempts have been made to characterize hemolymph (Summers, 1967; Nellaiappan et al., 1989) and cuticular PO (Ferrer et al., 1989a; Nellaiappan et al., 1982; Simpson et al., 1987; Savagaon and Sreenivasan, 1978) of crustaceans.

Phenol oxidases are copper-containing proteins which are generally believed to possess two enzymatic activities utilizing molecular oxygen: (1) monophenol hydroxylation ortho to the pre-existing hydroxyl group (mono-PO activity); and (2) *o*-diphenol oxidation to *o*-quinone (di-PO activity) (Mason, 1956). The reactions following the formation of *o*-quinone are believed to occur spontaneously (Lerner and Fitzpatrick, 1953) to form a leuco compound which is oxidized to the quinone state, dopachrome, followed by oxidation, decarboxylation, rearrangement, and polymerization to brownish black "melanin" pigments. This reaction causes a dark discoloration on the tails of lobsters during iced storage which lowers their quality and affects consumer acceptance (Ferrer et al., 1989a).

PO activity is usually determined by measuring dopachrome formation spectrophotometrically at 475 nm (Fling et al., 1963) or by following the formation of melanochrome (purple pigment) at 540 nm (Vachtenheim et al., 1985). Monophenol activity may be followed by measuring oxygen uptake either in a Warburg respirometer or by use of an oxygen-sensitive electrode. However, these techniques also measure oxygen uptake of *o*-diphenol oxidation. Whitaker (1972) has suggested that these methods may be inadequate and that a more specific method may be to follow the reaction spectrophotometrically at a wavelength where only *o*-diphenol formation is observed. Pomerantz (1964) studied the initial hydroxylation step of [3,5-^{3}H]-L-tyrosine by PO by measuring the rate of tritium released as water, which is directly proportional to the rate of hydroxylation.

Nagatsu et al. (1979) partially purified DOPA formed during the reaction by ion-exchange and aluminum oxide chromatography and quantified the amount present by reversed-phase HPLC with an electrochemical detector. However, Haavik and Flatmark (1980) separated DOPA and tyrosine using HPLC with a cation-exchange column of sulfonated fluorocarbon polymer coated on a pellicular silica support. Ferrer et al. (1989a) isolated three PO forms from the cuticle of the Florida spiny lobster, including an endogenously active PO. Using a spectrophotometric method to measure the rate of formation of dopachrome at 475 nm, they failed to observe mono-PO activity with tyrosine as a substrate. In the present study, the endogenously active PO from the cuticle of the spiny lobster was purified and the hydroxylation of L-tyrosine to DOPA monitored using ion-pair reversed-phase HPLC. The hydroxylation of tyramine (which has a similar structure to tyrosine but lacks a carboxyl group) by PO was also studied using a spectrophotometric method which measured the increase in absorbance at 280 nm. Tyrosine has been reported to be a natural substrate for PO activity in crustacea (Bailey et al., 1960b; Summers, 1967; Koburger et al., 1985), and the role of PO in the hydroxylation of tyrosine may be an important step in melanin synthesis. The objective of this study was to obtain a sensitive method to assay this activity and to acquire a better understanding of the properties and mechanism(s) of action of PO which would enable researchers to determine methods of controlling the deleterious effects of melanosis.

MATERIALS AND METHODS

L-Tyrosine, DL-DOPA, 1-octanesulfonic acid (sodium salt), and standards for electrophoresis were purchased from Sigma Chemical Co. Potassium phosphate monobasic (HPLC grade), glycine, and methanol (HPLC grade) were purchased from Fisher Scientific Co. Standards for isoelectric focusing were purchased from Pharmacia Inc. Florida spiny lobsters were obtained from the Florida Keys and maintained in tanks with flow-through seawater circulation at the Whitney Marine Laboratory, Marineland, FL. Lobster tails were frozen and transported in ice from Marineland to Gainesville, FL.

Phenol Oxidase Extraction. Cuticles were removed from the tails of lobsters (late premolt stage), washed with distilled water, and frozen in liquid nitrogen. Frozen cuticles were ground

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to a powder in a Waring blender and stored frozen at -20 °C until needed. Powdered cuticle was used to prepare crude extracts according to the method of Ferrer et al. (1989a).

Purification of Phenol Oxidase. Preparative nondenaturing polyacrylamide gel electrophoresis (PAGE) at 5% acrylamide and 0.13% bis(acrylamide) was used for initial purification of PO from the cuticular extract as described by Ferrer et al. (1989a). PO was detected using specific enzyme staining solution: 5 mM DOPA in 0.05 M sodium phosphate buffer (pH 6.5). Bands having PO activity were sliced from the gel, concentrated (50K Omega cell filter), rerun on preparative nondenaturing PAGE [7.5% acrylamide, 0.20% bis(acrylamide)], and repeated again to obtain a purified PO extract. The specific enzyme stain did not detect any other PO bands. Therefore, no multiple forms of the enzyme were observed.

Determination of Phenol Oxidase Activity with the Substrate DL-DOPA. PO activity was determined by the methods of Savagaon and Sreenivasan (1978) and Ferrer et al. (1989a). The rate of dopachrome formation was measured spectrophotometrically (Beckman DU-7) at 475 nm under the following conditions: 0.2 mL of enzyme extract was added to 2.8 mL of 10 mM DL-DOPA in 0.05 M potassium phosphate buffer (pH 6.5) at 23 °C, and the initial rate of increase in absorbance was determined.

Analytical Electrophoresis and Molecular Weight Determination. Analytical nondenaturing PAGE at 7% acrylamide and 0.19% bis(acrylamide) in the running gel with a 3% acrylamide stacking gel was carried out using a mini-Protean II dual slab cell (Bio-Rad Laboratories) with a slight modification (Bryan, 1977; Davis, 1964). Mini slab gels were 1 mm thick, and the sample size used was 15 μg. Electrophoresis was run at constant voltage (200 V) using a Pharmacia Model EPS 500/400 power supply. Chamber buffer was 0.01 M tris(hydroxymethyl)aminomethane/0.04 M glycine (pH 8.3).

MW was determined with SDS-PAGE according to the protocol of the Sigma Chemical Co. (1988). Since PO activity was not destroyed in the presence of SDS, electrophoresis was performed with and without 2-mercaptoethanol in the sample buffer which contained 0.1% SDS. When 2-mercaptoethanol was used in the sample buffer, the solution of PO and sample buffer was heated for 1 min at 100 °C. Tubes (0.5 cm i.d. by 12.5 cm in height) and mini slab gels were used at 7% SDS-PAGE with 3% stacking. Sample sizes were 30 μg for the tube gels, which were run at a constant current of 2 mA/tube, and 10-15 μg for the mini slab gels, which were run at constant voltage (200 V). MW standards were carbonic anhydrase (29 000), egg albumin (45 000), bovine albumin (66 000), phosphorylase B (subunit, 97 400), β-galactosidase (subunit, 116 000), and myosin (subunit, 205 000). PO bands were determined by an enzymatic staining assay using 0.05 M DOPA in 0.05 M sodium phosphate buffer (pH 6.5) followed by protein staining with a 0.25% solution of Coomassie brilliant blue R-250 in 40% methanol and 7% glacial acetic acid.

Isoelectric Focusing. Precast agarose isoelectric focusing gels with a pH range of 3-7 (FMC BioProducts) were used to determine the isoelectric point of PO. An isobox isoelectric focusing chamber with a Peltier cooling unit (Hoefer Scientific Instruments) was attached to a constant power supply at 500 V and a current of 20 mA. Focusing and Coomassie blue staining conditions were those described in the *Isogel* booklet (FMC BioProducts, 1986). Five-microgram samples of PO and a mixture of standards were applied to sample wells on the gels, and focusing was completed in approximately 1 h. The apparent isoelectric point (p*I*) of PO was calculated by comparison with standard proteins of known p*I* values using the p*I* focusing calibration kit of pH range 2.5-6.5 (Pharmacia). The gels were stained in a 0.1% solution of Coomassie brilliant blue R-250 in 25% ethanol and 9% glacial acetic acid.

Protein Determination. Protein content was determined by the biuret method (Clark and Switzer, 1977) at 540 nm.

Chromatographic Conditions. Tyrosine analysis by HPLC was a modification of that described by Van Boekel and Arentsen-Stasse (1987) and Feilchenfeld et al. (1982). An HP 1090 chromatograph equipped with an HP-85B computer and diode array detector (DAD) was used. The mobile phase was a mixture of 5% methanol in 0.1 M potassium phosphate buffer (pH 3.6) containing 0.5 mM sodium octyl sulfonate. A reversed-phase 10-μm C_{18} μ-Bondapak (Phenomenex) column (300 mm long × 3.9 mm i.d.) was used with a guard column [10 μm, C_{18} μ-Bondapak (Phenomenex), 30 mm long × 3.9 mm i.d.]. The columns were kept at 40 °C with a flow rate of 1.0 mL/min, and an injection volume of 20 μL was used. Detection of DOPA and tyrosine was by UV absorbance at 210 nm.

Enzyme Assay of Tyrosine Hydroxylation. Two hundred microliters of PO extract (3 mg/mL) was added to 2.8 mL of 1 mM L-tyrosine in 0.05 M phosphate buffer (pH 6.5). The mixture was incubated at 30 °C in a recirculating water bath with controlled temperature. Aliquots (0.5 mL) were removed at intervals and added to 0.5 mL of 0.1 M HCl containing 2 mM sodium ascorbate in methanol (4 °C) to stop the reaction and then immediately frozen. The mixture was subsequently thawed and filtered through a 0.45-μm membrane and quantitated by ion-pair reversed-phase HPLC. A control with heat-denatured enzyme instead of active PO was used.

pH Optimum. The optimum pH for tyrosine hydroxylation by PO was determined using 1 mM L-tyrosine in different buffers at concentrations of 0.05 M. The buffer solutions were sodium citrate-HCl (pH 4.0, 5.0, 5.5), potassium phosphate (pH 6.0, 6.5, 7.0, 7.5, 8.0), and glycine-NaOH (pH 9.0, 10.0). PO was then assayed (30 °C) as described previously, and the formation of DOPA was measured by ion-pair reversed-phase HPLC. Two replicates of the complete experiment were conducted.

pH Stability. pH stability was evaluated by incubating (30 min) on ice PO (5.3 mg/mL) with different buffers (0.1 M) at a ratio of 1:3 (PO:buffer) at the following pH values: sodium citrate (pH 2.0, 3.0, 4.0, 5.0), potassium phosphate (pH 6.0, 7.0, 8.0), and glycine (pH 9.0). Residual enzyme activity was measured by adding aliquots (200 μL) of incubated PO to 1 mM L-tyrosine (2.8 mL) and determining the rate of DOPA formation at 30 °C by ion-pair reversed-phase HPLC. Two replicates of the complete experiment were conducted.

Temperature Stability. Aliquots of PO in 0.05 M phosphate buffer (pH 6.5) were incubated in a recirculating water bath with controlled temperature for 30 min at 25, 30, 35, 40, 50, and 60 °C and then rapidly cooled in an ice bath for 15 min before assaying for residual activity with 1 mM L-tyrosine in 0.05 M phosphate buffer (pH 6.5) at 30 °C. The rate of DOPA formation was measured using ion-pair reversed-phase HPLC. Three replicates of the complete experiment were conducted.

Temperature Optimum. The substrate, 1 mM L-tyrosine in 0.05 M phosphate buffer (pH 6.5), was preincubated at various temperatures in a recirculating water bath with controlled temperature for 25 min prior to addition of the enzyme (PO) and the rate of DOPA formation at the different temperatures measured by ion-pair reversed-phase HPLC. Three replicates of the complete experiment were conducted.

Michaelis Constant for Tyrosine Hydroxylation. Michaelis constant (K_m) was determined from the Lineweaver-Burk reciprocal plot. The hydroxylation rate of tyrosine was determined as described above, using L-tyrosine in 0.05 M potassium phosphate buffer (pH 6.5) ranging in concentrations from 0.125 to 1 mM. Three replicates of the complete experiments were conducted.

RESULTS AND DISCUSSION

The specific activity of the purified PO, using DL-DOPA as substrate, was about 37-fold greater than that of the crude extract (Table 1). Savagaon and Sreenivasan (1978), using an ion-exchange procedure, reported a 3-fold purification of PO from the cuticle of spiny lobster (*Panulirus homarus* Linn.).

Analytical nondenaturing PAGE showed one band using the enzyme staining method and the same band occurred when Coomassie blue (Figure 1A) was used to stain for protein. In the presence of 2-mercaptoethanol (SDS-PAGE) in the sample buffer (Figure 1B), one major protein band of MW 81 200 ± 500 was obtained, which corresponded to the activity band obtained without 2-mercaptoethanol in the sample buffer.

Table 1. Purification Scheme for Phenol Oxidase from the Cuticle of the Florida Spiny Lobster[a]

step	total vol, mL	total protein, mg	total act., EU[b]	sp act., EU/mg of protein	yield, %	purifn
crude	50.0	1600.0	160.0	0.1	100.0	1.0
preparative PAGE (5%)	20.0	73.2	81.3	1.1	50.8	11.1
preparative PAGE (7.5%)	2.0	6.0	22.2	3.7	13.9	37.0

[a] Data presented are representative of most trials. [b] EU (enzyme units) are expressed as micromoles of DL-DOPA per minute.

A wide range of values for the MW of PO have been reported in the literature. Nellaiappan et al. (1989) reported apparent MW in the range of 42 000–350 000 for different crustaceans. The MW of PO from heads of white shrimp (*Penaeus setiferus*), purified by affinity chromatography and determined by SDS–PAGE, was 30 000 (Simpson et al., 1987), which was within the range reported for PO isozymes in kiwi fruit (Park and Luh, 1985). However, the MW from *Penaeus aztecus* and *Xyphopenaeus kroyerii*, determined by gel filtration by Madero and Finne (1982), were 213 000 and 76 000, respectively. Vinayakam and Nellaiappan (1987) found four PO isozymes in the cuticle of postmolt *Emerita emeritus* with a range of MW from 25 000 to 140 000 and two isozymes at the intermolt stage of MW 140 000 and 50 000. They suggested the possibility of aggregation to higher MW forms was caused by activation of PO during the molt stages.

In insects, subunit MW of PO ranged between 29 000 and 36 000 (Ashida, 1971; Pau and Eagles, 1975), and the MW 36 000 subunit readily dimerizes to give MW of 72 000 or 80 000 by ultracentrifugation and electrophoresis, respectively (Ashida, 1971). Pau and Eagles (1975) reported that an active PO from *Calliphora* having a MW of 210 000 was a hexamer. Ferrer et al. (1989a) obtained a MW, determined by nondenaturing PAGE, of 62 500 for active PO extracted from the cuticle of the Florida spiny lobster; however, the MW obtained in the present study was 81 200.

The p*I* obtained for PO was 4.76 ± 0.03. This value was within the range of p*I* values obtained for PO from different sources. Thomas and Janave (1986), working with a partially purified enzyme preparation from pulp tissue of unripe Dwarf Cavendish bananas, revealed 14 PO isozymes with p*I*s in the pH range 4–5.5 and two strains of the Red Skin showed 10 and 13 isozymes with p*I*s in the pH range 4–5.8. Galeazzi et al. (1981) showed a p*I* value of 5.2 for PO extracted from banana pulp. Janovitz-Klapp et al. (1989) obtained two major bands of PO activity with p*I*s of 4.5 and 4.8 from Red Delicious apple. The granular PO responsible for cuticular melanization in *Manduca sexta* larva was found to consist of four isozymes with p*I*s ranging from 5.7 to 5.85.

Mono-PO activity from lobster cuticle has not been demonstrated before. This could be attributed to inadequate methods of measuring the hydroxylation of monophenols. Ferrer et al. (1989a) reported only di-PO activity in three PO forms isolated from the cuticle of the Florida spiny lobster including the endogenously activated PO used in the present study. These forms acted on DL-DOPA but not on the monophenol, tyrosine. Attempts to determine the K_m value with tyrosine as substrate failed when a spectrophotometric method was used. It has been reported (Brunet, 1980) that PO from the cuticle of insects does not exhibit mono-PO activity but hemolymph PO does. The diphenols needed for the cuticle PO to form the tanning quinones may be produced in the hemolymph of the insect and transported to the cuticle. It has been suggested that a similar mechanism may occur in lobsters since both lobsters and insects use PO in the sclerotization process (Ferrer et al., 1989b).

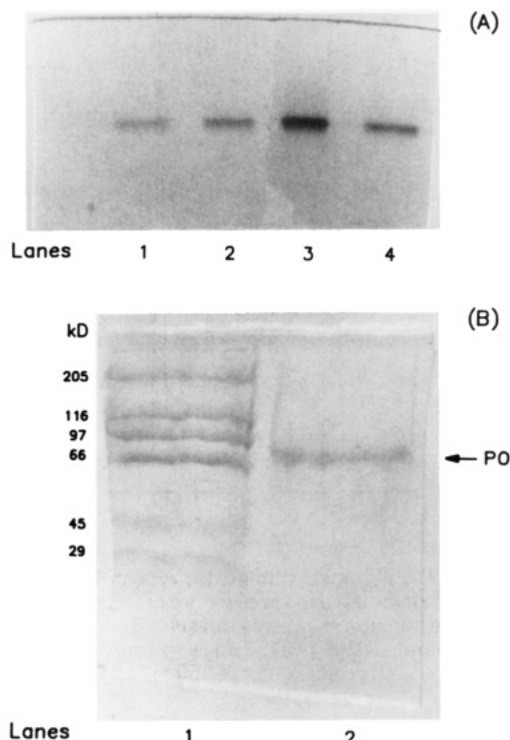

Figure 1. Analytical PAGE of PO: (A) nondenaturing PAGE (7%), (lanes 1 and 2) PO (DOPA stained), (lanes 3 and 4) PO (Coomassie blue stained); (B) SDS–PAGE (7%), Coomassie blue stained, (lane 1) molecular weight standards, (lane 2) PO with 2-mercaptoethanol in the sample buffer.

Crustacean cuticles contain phenolic compounds that are presumed to be substrates of the enzymes involved in sclerotization (Stevenson, 1963). Since tyrosine has been reported to be a natural substrate for PO activity in shrimp (Bailey et al., 1960a; Summers, 1967) and spiny lobster (Koburger et al., 1985), a rapid, simple, and sensitive method for the assay of mono-PO activity in crustacean cuticle is needed. Ion-pair reversed-phase HPLC demonstrates that PO isolated from the cuticle of the Florida spiny lobster can catalyze the hydroxylation of tyrosine to DOPA (Figure 2)

DOPA formed as a product of the hydroxylation reaction is well separated from the residual tyrosine. The concentration of DOPA formed at the initial reaction rate was very low compared to the tyrosine concentration. Peak areas were calculated manually and converted to concentration units using a standard curve. A linear relationship was obtained between the amount of DOPA injected and the peak area ($r^2 = 0.99$); the lower limit of detection was about 15 pmol of DOPA (data not shown).

Each compound was identified by its retention time and/or by enriching with standards under the same conditions. The reproducibility of retention times was very good with low standard deviations.

It has been suggested that when a monophenol is added alone as substrate, there is a lag period due to the need for an *o*-diphenolic compound to build up in sufficient amount to permit the reaction to proceed (Whitaker, 1972). The time curve (Figure 3) for the PO-catalyzed hydroxylation of 0.05 M tyrosine in phosphate buffer (pH 6.5), at 30 °C shows a lag time of approximately 7 min. In the present study, an *o*-diphenolic compound was not added.

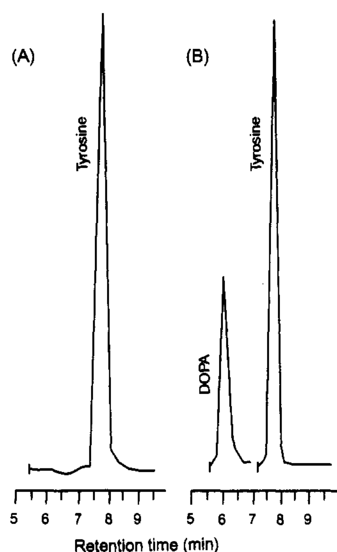

Figure 2. HPLC chromatograms of tyrosine in 0.05 M phosphate buffer (pH 6.5) incubated in the presence of PO at 30 °C. Aliquots were sampled at intervals using reversed-phase HPLC. (A) 0 min; (B) 20 min. DOPA peak: range = 5 mAU; peak width = 0.29, min. Tyrosine peak: Range = 150 mAU; peak width = 0.5 min.

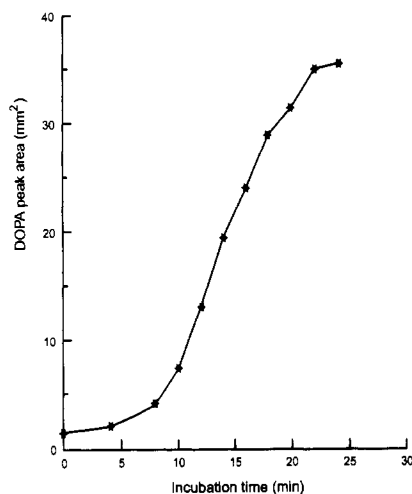

Figure 3. Time course of PO-catalyzed tyrosine hydroxylation (HPLC). An aliquot of 200 µL (3 mg/mL) of PO was added to 2.8 mL of 1 mM tyrosine in 0.05 M phosphate buffer (pH 6.5), incubated at 30 °C and sampled at intervals. The rate of DOPA formation was measured by ion-pair reversed-phase HPLC.

Pomerantz (1964) suggested that the lag can be shortened, but usually not eliminated, by catalytic amounts of diphenol. Only initial rates of DOPA formation were used in the experiments to eliminate interference from later polymerization reactions which occur. Since catechols are extremely susceptible to autoxidation (Ito et al., 1984), sodium ascorbate was included in the stopping solution which served as a trapping reagent to reduce any quinones formed. A control using heat-denatured enzyme did not catalyze the formation of DOPA, indicating that the reaction was tyrosinase dependent.

pH Optimum and Stability of PO. The pH optimum obtained for the hydroxylation of tyrosine by PO was between pH 6 and 6.5 (Figure 4A), and the enzyme was most stable from pH 6.0 to 8.0 (Figure 4B), losing only 12.5% of its original activity at pH 9. The PO was not stable at acidic pH values.

Ferrer et al. (1989a) reported an optimum pH of 8.0 for endogenously activated PO using DL-DOPA as substrate; they also noted that the PO was unstable below pH 4.0

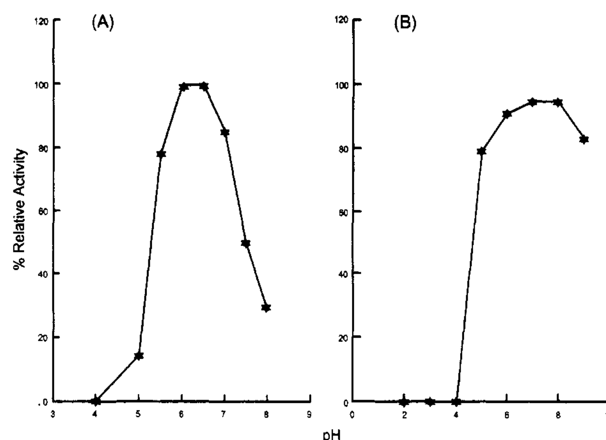

Figure 4. (A) Optimum pH of PO-catalyzed hydroxylation of tyrosine. Aliquots of 200 µL (3 mg/mL) of PO were added to 2.8 mL of 1 mM tyrosine in buffers of different pH values as described under Materials and Methods. The mixtures were incubated at 30 °C and sampled at intervals. The rate of DOPA formation, at each pH value, was determined by ion-pair reversed-phase HPLC. (B) pH stability curve of PO. Samples of PO stock solution in distilled water (5.3 mg/mL) were mixed 1:3 (v/v) with 0.1 M buffer solutions of pH values (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0) and incubated on ice for 30 min. Residual enzyme activity was determined with 1 mM tyrosine in 0.05 M phosphate buffer (pH 6.5) at 30 °C and quantitated by ion-pair reversed-phase HPLC. The activity was calculated relative to the maximum activity.

and had a pH stability range of 6.0–10.0. These results on pH stability of PO correlate well with the results obtained in the present study.

The values reported in the literature for optimum pH activity for PO vary widely. Factors such as source of the enzyme, substrate used, and buffer systems can affect the optimum value. In a number of studies, optima between pH 6.0 and 8.0 have been reported, but in some cases activity may continue to increase or to remain at a high value up to pH 9.5. In general, the activity is progressively decreased as the pH is lowered below pH 6.0 (Faulkner et al., 1954).

Simpson et al. (1987) purified PO from the heads of white shrimp and found that the enzyme, using DL-DOPA as substrate, was most active between pH 6.5 and 7.5 and was most stable at pH 8. Similar pH activity was reported for other POs such as kiwi fruit isozymes with optimum pH of 6.8 and 7.3 with catechol as substrate and pH 7.5 and 8.0 when (+)-catechin was used as substrate (Park and Luh, 1985).

Temperature Optimum and Stability of PO. The PO was stable up to 40 °C (Figure 5A), but after incubation at 60 °C for 30 min, the enzyme was completely inactivated. Ferrer et al. (1989a) reported that crude preparations of the PO were stable at temperatures below 35 °C and lost activity at 60 °C. The optimum temperature for tyrosine hydroxylation was 40 °C under the conditions used in this study (Figure 5B). Simpson et al. (1987) found that the optimum temperature for PO from white shrimp was 45 °C and that it was stable up to 50 °C. Total inactivation of PO from shrimp head extracts occurred at 50 °C (Madero and Finne, 1982); however, deep sea crab PO appeared to be slightly more heat stable, and total inactivation occurred at 70 °C (Marshall et al., 1984).

Since the "temperature optimum" of an enzyme is an operational parameter rather than a true characteristic (Whitaker, 1972) and stability of an enzyme is a function not only of temperature but also of a number of factors including pH, ionic strength, nature of the buffer, and time of incubation, it is difficult to compare data unless the conditions are similar. However, it appears that the

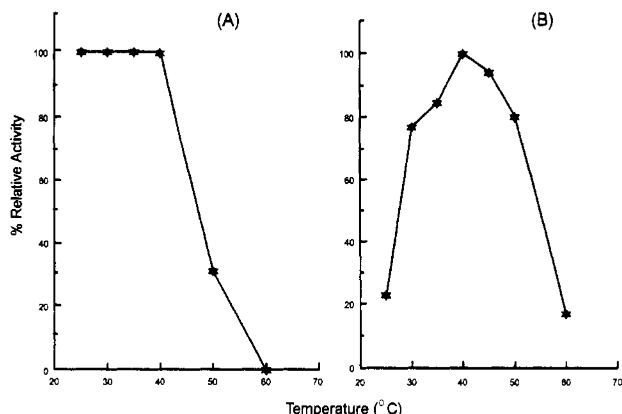

Figure 5. (A) Temperature stability curve of PO. Aliquots of PO (3 mg/mL) in 0.05 M phosphate buffer (pH 7.2) were incubated for 30 min at 25, 30, 35, 40, 50, and 60 °C and rapidly cooled on ice for 15 min. Residual activity was determined with 1 mM tyrosine in 0.05 M phosphate buffer (pH 6.5) at 30 °C. The rate of DOPA formation was measured using ion-pair reversed-phase HPLC. (B) Optimum temperature of PO-catalyzed hydroxylation of tyrosine. Samples of 1 mM tyrosine in 0.05 M phosphate buffer (pH 6.5) were pre-equilibrated at the following temperatures for 25 min: 25, 30, 35, 40, 45, 50, and 60 °C. Aliquots of 200 μL of PO (3 mg/mL) were added to 2.8 mL of tyrosine at each temperature, and the rate of DOPA formation was measured by ion-pair reversed-phase HPLC. The activity was calculated relative to the maximum activity.

PO from the cuticle of the Florida spiny lobster shows some similarity to PO from other sources.

Michaelis Constant (K_m). K_m values were obtained with tyrosine as substrate using ion-pair reversed-phase HPLC. The apparent K_m was 0.97 ± 0.05 mM with tyrosine as substrate. Ferrer et al. (1989a) obtained an apparent K_m of 0.92 mM for PO with DL-DOPA as substrate. Summers (1967), using PO from the fiddler crab and following hydroxylation of monophenols by measuring a decrease in absorbance at 250 nm, found that tyramine had a K_m about 6000 times smaller than that of tyrosine. They also determined a similar relationship between DOPA and dopamine and concluded that the carboxyl group attached to the side chain or directly to the aromatic nucleus tends to inhibit the action of the enzyme. Pomerantz (1964) used [3,5-^{3}H]-L-tyrosine for studying the initial step catalyzed by tyrosinase and found that the rate of tritium released as water was directly proportional to the rate of hydroxylation. The K_m for tyrosine by this method was 0.3 mM as compared to 0.6 mM by dopachrome measurement.

There have always been questions of whether the same or different enzymes catalyze the oxidation of mono- and dihydric phenols. Mason (1955) suggested that cresolase (mono-PO) and catecholase (di-PO) differed in substrate specificity and catalyzed the oxidation/reduction reactions but had a single active site. However, Dressler and Dawson (1960) reported that there were two active sites in the PO molecule. Park and Luh (1985), working on kiwi fruit POs concluded that catecholase and cresolase have different active sites. Jolley and Mason (1965) suggested that the existence of two unlike subunits may explain the differences observed among the multiple forms of PO toward mono- and diphenols. They also noted that the mushroom isozymes were to a certain degree interconvertible, depending on pH, ionic strength, and protein concentration. Bailey et al. (1960b) showed that shrimp PO catalyzed the reactions of both mono- and dihydric phenols; they suggested that there is a close similarity of PO from various sources and that a single enzyme is involved in both mono- and di-PO activities.

Conclusion. The endogenously active PO from the cuticle of the Florida spiny lobster was shown to have both mono- and di-PO activity. The use of HPLC facilitated the identification and quantitation of DOPA formation when a spectrophotometric method, as reported by Ferrer et al. (1989a), failed to determine mono-PO activity with tyrosine as substrate. The short analysis time was facilitated by the direct determination of the actual enzymatic reaction product (DOPA); since no chemical derivatization of DOPA was necessary, aliquots of the assay solution were injected directly onto the HPLC column. The high sensitivity of the detector made it possible to quantitate very low product concentration.

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