Comparative Studies on the Polyphenol Oxidase Fraction from Lobster and Tyrosinase

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Polyphenol oxidase (EC 1.10.3.1) fraction was extracted from the skin layer between the muscle and the exoskeleton of lobster (*Homarus americanus*), and its properties were compared with those of commercial tyrosinase. The lobster PPO fraction was activated by trypsin, and its pH optimum for the oxidation of Dopa was determined as 6.5. The lobster enzyme was most stable at pH 7.5, while tyrosinase exhibited a much broader pH stability range, from 6.5 to 10.0. The temperature optimum for the oxidation of Dopa was 30 °C with the lobster enzyme and 40 °C with tyrosinase. The lobster enzyme was more heat labile as compared with commercial tyrosinase. While the lobster enzyme lost most than 50% of its original activity after 30 min of incubation at 35 °C, tyrosinase retained approximately 90% of its original activity after 30 min of incubation at 55 °C. The activities of both enzymes were enhanced by copper but inhibited by cysteine, *p*-aminobenzoic acid, and EDTA.

INTRODUCTION

Polyphenol oxidases (PPO) catalyze the dark discolorations or melanosis in fruits, vegetables, and crustacean species (Ferrer et al., 1989; Simpson et al., 1987; Savagaon and Sreenivasan, 1978). The phenomenon of melanosis has been extensively studied in insects, fruits, and vegetables (Brunet, 1980; Wong et al., 1971) but has not received as much attention in economically important crustacean species such as crab, shrimp, and lobster. These post-mortem dark discolorations reduce the quality and acceptability of crustacean species, and this defect has stimulated efforts aimed at finding effective treatments for melanosis in these species. Fundamental studies aimed at obtaining a better understanding of melanosis are needed to provide the information needed for rationalizing and/or formulating handling and process manipulations to improve the postharvest quality and acceptability of these species. Investigations carried out by various researchers (Ferrer et al., 1989; Simpson et al., 1988; Koburger et al., 1985; Soderhall and Hall, 1984; Marshall et al., 1984; Savagaon and Sreenivasan, 1975) have indicated the following: (i) PPO from the cuticle of the Florida spiny lobster (*Panulirus argus*) occurs in a precursor form which activates on storage or with the aid of trypsin into three active forms; (ii) the level of substrates for melanosis varies from one species to another and may be a contributory factor in the different degrees of melanosis elicited by different crustacean species; (iii) melanosis is initiated from the epidermis and migrates into muscle tissue; (iv) liposaccharides from *Escherichia coli* and *Salmonella abortus* activate a proteolytic enzyme which, in turn, potentiates the activation of the zymogen form of PPO; and (v) a relationship exists between PPO levels, season, and molting stage. Recently, McEvily et al. (1991) described the use of 4-hexylresorcinol for retarding melanosis in brown shrimp (*Penaeus aztecus*) and pink shrimp (*Penaeus duorarum*). In the present study PPO fraction was isolated from lobster and characterized with respect to its kinetic properties and responses to pH, temperature, and inhibitors. It is anticipated that such information may be useful in evolving alternative methods for controlling these dark discolorations in the post-mortem animal.

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MATERIALS AND METHODS

Materials. Live lobsters (*Homarus americanus*) were obtained from a local market and "butchered" to recover the skin layer between the muscle and exoskeleton for use as source of the PPO. This source material was comminuted to fine powder with liquid nitrogen in a Waring blender and stored at −80 °C until use. Dihydroxyphenylalanine (Dopa), *p*-aminobenzoic acid (PABA), tyrosinase, and trypsin (bovine pancreas, type III) used in the study were purchased from Sigma Chemical Co. Ethylenediaminetetraacetic acid (EDTA) and cysteine were purchased from Fisher Scientific Co. and Nutritional Biochemicals Corp., respectively.

Extraction of PPO. The extraction process involved stirring the frozen powder in 0.05 M sodium phosphate buffer (pH 7.2, containing 0.5 M NaCl and 2 μg/mL trypsin) for 4 h at 4 °C. The ratio of lobster powder to extraction buffer was 1:10 (w/v). The homogenate resulting from the stirring was centrifuged at 15000*g* for 30 min at 4 °C, and the clear supernatant was fractionated with solid ammonium sulfate. The fraction precipitating between 40 and 80% saturation was collected by centrifugation at 15000*g* for 30 min at 4 °C, redissolved in 0.05 M sodium phosphate buffer (pH 7.2, containing 0.5 M NaCl), and then dialyzed overnight against three changes of 4 L of 5 mM sodium phosphate buffer (pH 7.2). The dialyzed extract was designated PPO fraction and stored at −20 °C until use.

Assay of PPO Activity. The activity of PPO fraction or tyrosinase was determined spectrophotometrically using a modified form of the procedure of Horowitz et al. (1960) with Dopa (5 mM, in 0.05 M phosphate buffer, pH 6.5) as substrate. The reaction mixture comprised 2.8 mL of the substrate solution and 0.2 mL of enzyme (or buffer as blank). A unit of enzyme activity was defined as the change in absorbance of 0.001/min at 475 nm and 25 °C. Protein was determined according to the method of Hartree (1972) using bovine serum albumin as standard.

Influence of Trypsin on PPO Activity. The influence of trypsin on the activity of PPO in the crude extract was determined using various concentrations of trypsin. The enzyme fraction was incubated with or without trypsin for 30 min before activity assays were performed as described above.

pH Optimum and Stability. The influence of pH on activity of PPO fraction or tyrosinase was determined by preparing the substrate, Dopa (5 mM), in various buffer solutions and applying

0021-8561/92/1440-0772$03.00/0

Table I. Scheme for Recovery of Lobster PPO Fraction[a]

step	total vol, mL	total protein, mg	total act., units	sp act., units/mg	yield, %
crude extract	155	3509	5881	1.7	100
$(NH_4)_2SO_4$ extract 1[b]	160	1761	3253	1.8	55.3
extract 2[c]	16	212	4464	21.1	75.9

[a] Fifteen grams of powdered lobster skins was treated as described under Materials and Methods. Data are representative of two other trials. [b] Fraction remaining after removing 40% $(NH_4)_2SO_4$ precipitate by centrifugation. [c] Extract prepared by dissolving 40–80% $(NH_4)_2SO_4$ precipitate in extraction buffer.

aliquots of the enzyme solution individually to the buffered substrate solution. Enzyme activity was determined by measuring the initial rate of formation of dopachrome at 475 nm and 25 °C. The compositions of the buffer solutions used were as follows: 0.1 M KCl–HCl, pH 2.0; 0.1 M citrate–NaOH, pH 4.0; 0.1 M citrate–NaOH, pH 6.0; 0.1 M sodium phosphate, pH 6.5; 0.1 M sodium phosphate, pH 7.0; 0.1 M sodium phosphate, pH 7.5; 0.1 M sodium phosphate–NaOH, pH 8.0; 0.1 M Tris-HCl, pH 9.0; 0.1 M glycine–NaOH, pH 10.0; 0.1 M glycine–NaOH, pH 12.0.

The influence of pH on the stability of activated PPO fraction or tyrosinase was determined by preincubating the enzymes with the buffer solutions described above (ratio of enzyme stock solution to buffer = 1:1 v/v) at 25 °C for 30 min. Residual enzyme activity was assayed with Dopa (5 mM, pH 6.5) as substrate by measuring the initial rate of formation of dopachrome at 475 nm and 25 °C. In both cases, control experiments were run without PPO.

Temperature Optimum and Stability of PPO. The effect of temperature on the activity of PPO fraction or tyrosinase was determined with Dopa (5 mM in 0.05 M phosphate buffer, pH 6.5) as substrate at various temperatures. The substrate solutions were pre-equilibrated at various temperatures (15–70 °C) at 5 °C intervals for 10 min prior to addition of enzyme. The rate of dopachrome formation was assayed as described previously (Simpson et al., 1987). The temperature stability of the enzymes was investigated by incubating the enzymes at various temperatures (25–90 °C) at 10 °C intervals for 30 min. The heat-treated enzyme solutions were rapidly cooled in an ice bath prior to assay for residual activity with Dopa (5 mM in 0.05 M phosphate buffer, pH 6.5) as substrate. A control experiment was also run without PPO.

Kinetic Properties (K_m' and V_{max}). Solutions of Dopa ranging in concentration from 2 to 10 mM (Ferrer et al., 1989; Simpson et al., 1988) were employed to study the kinetic properties of PPO and commercial tyrosinase at 475 nm and pH 6.5. The Michaelis constants (K_m') and substrate turnover numbers (V_{max}) were calculated by least-squares analysis from Lineweaver–Burk plots as described by Park and Luh (1985), Fujita and Tono (1988), and Ferrer et al. (1989).

Response of PPO to Inhibitors. The influence of various inhibitors (cysteine, EDTA, and PABA) on the activity of PPO fraction or tyrosinase was investigated as follows: To 1 mL of approximately 25 μg/mL PPO fraction or 48 μg/mL tyrosinase were added various aliquots of 5 mM cysteine, 5 mM EDTA, or 5 mM PABA in 0.05 M phosphate buffer (pH 6.5) as specified in Table III. The enzyme–inhibitor solutions were incubated at 25 °C for 30 min, and the residual PPO activity was assayed with Dopa (5 mM, pH 6.5) as substrate at 475 nm and 25 °C. Control experiments, without inhibitors, were run simultaneously.

Effect of Copper on PPO Activity. The effect of copper on the activity of PPO was determined using a modified form of the procedure of Bailey et al. (1959). It involved incubating the enzyme solution with or without copper acetate (1:1) in an ice bath for 30 min prior to PPO activity assay at 475 nm and 25 °C as described previously.

RESULTS AND DISCUSSION

A summary of the purification scheme for lobster PPO fraction is presented in Table I. The specific activity of the precipitate resulting from 40–80% ammonium sulfate fractionation increased about 12-fold as compared with that of crude extract. This represented a 75.9% recovery of enzyme activity. The yield of PPO fraction was about 212 mg of protein/15 g of raw material.

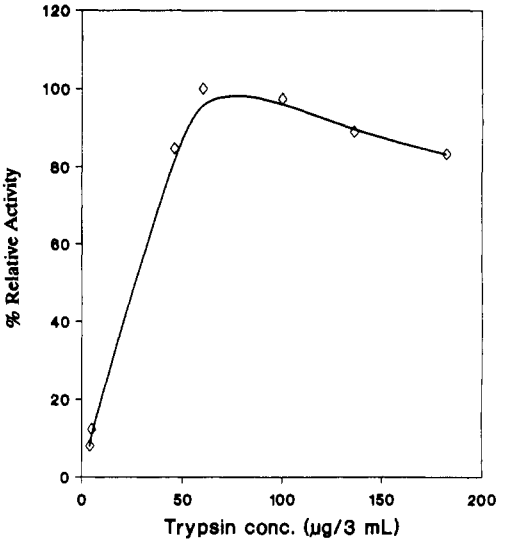

Figure 1. Influence of trypsin on lobster PPO fraction. Data used to plot figure are average values of triplicate results for two experiments. Protein concentration of stock enzyme solution was ~25 μg/mL.

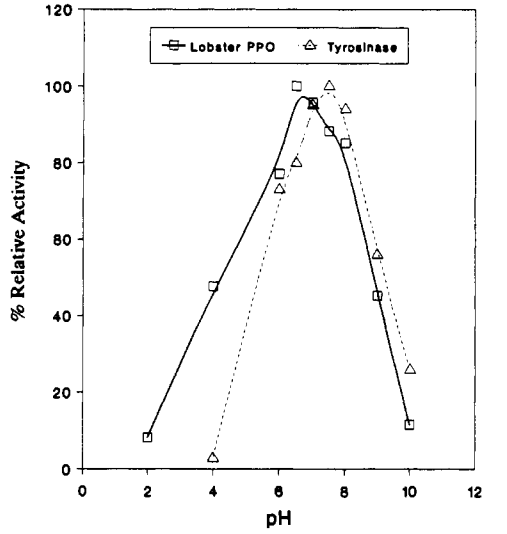

Figure 2. pH optima for oxidation of Dopa with lobster PPO fraction and tyrosinase. Data used to plot figure are average values of triplicate results for two experiments. Protein concentrations of stock enzyme solutions were ~25 (PPO fraction) and 48 μg/mL (tyrosinase). The pooled estimate of the standard deviation was ±4.2%.

Influence of Trypsin on PPO Activity. The influence of trypsin on PPO activity is summarized in Figure 1 and indicates that the activity of PPO was enhanced by trypsin. The enzyme activity increased with increasing concentration of trypsin up to 60 μg of trypsin/3 mL of the reaction mixture and then declined. This finding agrees with those made by Savagaon and Sreenivasan (1978) and Ferrer et al. (1989) and suggests that the lobster enzyme exists in a precursor form which requires further activation by trypsin or trypsinlike enzymes.

Influence of pH on Activity and Stability of PPO. The pH–activity profiles for the oxidation of Dopa are presented in Figure 2 and indicate that the PPO fraction was most active at pH 6.5, while commercial tyrosinase was most active at pH 7.5. This observation is consistent with those made by Wong et al. (1971) for peach PPO (pH

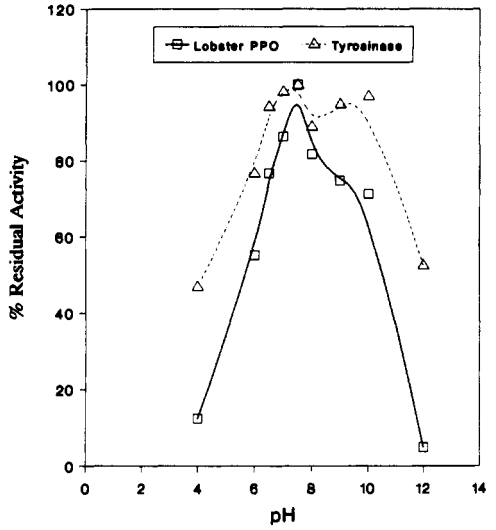

Figure 3. pH stability of lobster PPO fraction and tyrosinase. Data used to plot figure are average values of duplicate results for two experiments. Protein concentrations of stock enzyme solutions were ~25 (PPO fraction) and 48 μg/mL (tyrosinase). The pooled estimate of the standard deviation was ±4.7%.

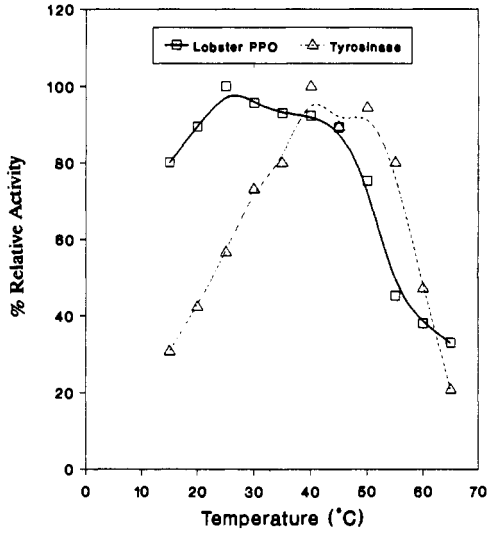

Figure 4. Temperature optima for oxidation of Dopa with lobster PPO fraction and tyrosinase. Data used to plot figure are average values of triplicate results for two experiments. Protein concentrations of stock enzyme solutions were ~25 (PPO fraction) and 48 μg/mL (tyrosinase). The pooled estimate of the standard deviation was ±3.8%.

optimum 7.0) and by Chan and Yang (1971) for cranberry PPO (pH optimum 7.0). The lobster enzyme was most stable at pH 7.5, while tyrosinase exhibited a much broader pH stability range (pH 6.5–10.0) as shown in Figure 3. The findings from the pH stability study are also similar to those reported elsewhere for PPO from various sources. For example, Simpson et al. (1988) reported that pink shrimp PPO was most stable over a broader pH range (6.5–9.0), while its white shrimp counterpart was most stable from pH 6.0 to 7.5. Savagaon and Sreenivasan (1975) also found different forms of PPO in lobster (*Panulirus homarus* Linn.) to be stable between pH 6.5 and 9.0.

Influence of Temperature on Activity and Stability of PPO. The influence of temperature on the activity of lobster PPO fraction and tyrosinase is presented in Figure 4. The data in Figure 4 indicate that the temperature optimum for the oxidation of Dopa by lobster PPO was 30 °C, while that of the tyrosinase–Dopa reaction was 40

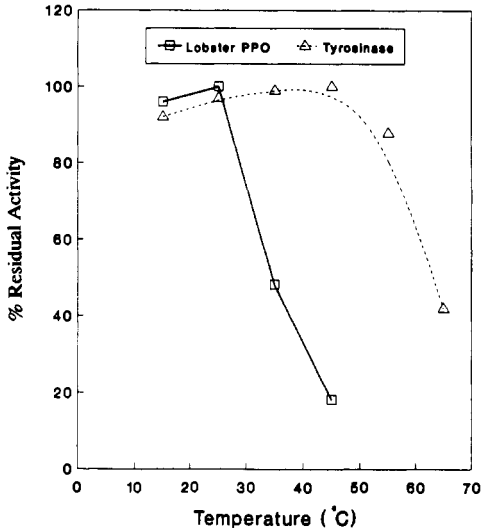

Figure 5. Temperature stability of lobster PPO fraction and tyrosinase. Data used to plot figure are average values of duplicate results for two experiments. Protein concentrations of stock enzyme solutions were ~25 (PPO fraction) and 48 μg/mL (tyrosinase). The pooled estimate of the standard deviation was ±5.6%.

Table II. Kinetic Parameters[a]

parameter	PPO fraction	tyrosinase
K_m', mM	2.13 ± 0.6	0.57 ± 0.4
V_{max}, units/min	55.0 ■ 1.2	111.0 ± 2.0
V_{max}/K_m', (units/min)/mM	25.8 ± 2.0	194.7 ± 5.0

[a] Data are average values of triplicate determinations for two experiments. The protein concentrations of stock enzyme solutions were ~25 (lobster PPO fraction) and 48 μg/mL (tyrosinase).

°C. A temperature optimum of 45 °C has been reported for the white shrimp phenol oxidase–Dopa reaction (Simpson et al., 1987) and also for an isoenzyme of *P. homarus* PPO–catechol (Savagaon and Sreenivasan, 1975). The thermostability data presented in Figure 5 indicate that lobster PPO was more heat labile than commercial tyrosinase. For example, the lobster enzyme fraction lost more than 50% of its original activity after 30 min at 35 °C, while tyrosinase retained about 90% of its original activity after 30 min of incubation at 55 °C.

Kinetic Properties (K_m' and V_{max}). The kinetic properties (K_m' and V_{max}) for the PPO fraction and commercial tyrosinase are presented in Table II. The K_m' for the oxidation of Dopa was higher for the reaction catalyzed by PPO than for that catalyzed by commercial tyrosinase. The substrate turnover number (V_{max}) for the lobster PPO–Dopa reaction was lower than that of the tyrosinase–Dopa reaction (Table II). The catalytic efficiency (V_{max}/K_m') for the lobster PPO–Dopa reaction was lower than that of the tyrosinase–Dopa reaction. The ratio V_{max}/K_m', which is a measure of the catalytic efficiency and/or substrate specificity of an enzyme (Whitaker, 1972), suggests that the commercial tyrosinase appears to be better suited to catalyze oxidation of Dopa than the lobster PPO.

Influence of Inhibitors on PPO Activity. The effect of various inhibitors on the activity of lobster PPO fraction and commercial tyrosinase is presented in Table III. Oxidation of Dopa by the lobster enzyme or tyrosinase was completely inhibited by cysteine. Both enzymes were inhibited by PABA, with the inhibitory effect being greater for lobster PPO fraction than tyrosinase. Furthermore, the activities of the two enzymes were inhibited by EDTA. However, unlike the situation observed with PABA,

Table III. Influence of Inhibitors on Activity of PPO Fraction and Tyrosinase[a]

inhibitor	inhibitor concn, mM	% inhibition	
		PPO fraction	tyrosinase
cysteine	3.3	100.0 ± 1.8	99.1 ± 0.7
	2.5	99.2 ± 1.1	98.8 ± 0.4
PABA	3.3	41.9 ± 2.3	73.8 ± 1.3
	2.5	28.5 ± 0.8	53.3 ± 2.5
EDTA	3.3	50.2 ± 1.6	42.3 ± 0.9
	2.5	47.6 ± 2.2	27.1 ± 1.2

[a] Data are average values of triplicate determinations for two experiments. The protein concentrations of enzyme solutions used in study were ~25 (lobster PPO fraction) and 48 μg/mL (tyrosinase). Percent inhibition values were calculated relative to control values.

Table IV. Effect of Copper on PPO Activity[a]

Cu concn, mM × 10^{-7}	% relative act.	
	PPO fraction	tyrosinase
0.0	100.0 ± 0.1	100.0 ± 0.1
0.5	140.5 ± 0.1	138.7 ± 0.3
5.0	141.5 ± 0.2	153.8 ± 0.2
50.0	152.9 ± 0.1	171.8 ± 0.2

[a] Data are average values of triplicate determinations for two experiments. The protein concentrations of enzyme solutions used in study were ~25 (lobster PPO fraction) and 48 μg/mL (tyrosinase). Percent relative activity values were calculated relative to control values.

inhibition was greater for tyrosinase than for PPO fraction. The susceptibility of both lobster PPO fraction and tyrosinase to inhibition by these compounds suggests that the two enzymes have similar mechanisms of substrate bonding by their active sites.

Effect of Copper on PPO Activity. The effect of copper on the activity of lobster PPO and tyrosinase is summarized in Table IV, which shows that the activities of the enzymes were stimulated by the addition of copper. This finding suggests that both the lobster enzyme and commercial tyrosinase are copper-dependent. This observation on copper dependency of PPO is also supported by the inhibition of this enzyme by EDTA. Copper dependency of PPO from various sources has been observed by various workers. For example, Simpson et al. (1987) reported a copper dependency of polyphenol oxidases from pink and white shrimp.

Conclusion. The study indicates that lobster PPO is similar to commercial tyrosinase with respect to substrate specificity and responses to pH and selected inhibitors (cysteine, PABA, and EDTA). However, the two enzymes differed from one another in terms of their thermal and pH stabilities. The study further indicates that lobster PPO occurs in a precursor form which becomes activated by trypsin to the active form. Further, the active PPO fraction is copper-dependent. Further studies in our laboratory have resolved the PPO fraction into three active components on the basis of their p*I* differences, and these three isozymes are being characterized with respect to their structural and functional properties. It is anticipated that the knowledge gained from these studies will facilitate the development of other alternative procedures for controlling melanosis in crustacean species.

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Received for review September 17, 1991. Accepted February 14, 1992.

Registry No. PPO, 9002-10-2; DOPA, 59-92-7; Cu, 7440-50-8.