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Enzymatic Characterization and Functional Groups of Polyphenol Oxidase from the Pupae of Blowfly (*Sarcophaga bullata*)

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Abstract—Polyphenol oxidase (EC 1.14.18.1) was purified from the pupae of blowfly (*Sarcophaga bullata*) by a procedure involving ammonium sulfate fractionation and chromatography on DEAE-cellulose and Sephadex G-100. Kinetic characteristics of the enzyme were determined using L-DOPA as substrate. The specific activity of the enzyme was 770 U/mg, and the Michaelis constant (K_m) was 1.5 ± 0.1 mM (pH 6.8, 30°C). Activity was maximal at 40°C, pH 6.5. Chemical modification experiments demonstrated that cysteine and tryptophan residues are essential and arginine residues are not essential to the enzyme function. The enzyme is inhibited by quercetin with an IC_{50} of 0.20 ± 0.06 mM. The inhibition is of competitive type, and the inhibition constant was determined to be 88 μM.

Key words: polyphenol oxidase, blowfly pupae, kinetic characterization, chemical modification

Polyphenol oxidase (EC 1.14.18.1, PPO) is the key enzyme involved in melanin formation in melanocytes. It catalyzes the *o*-hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (hydroxylase activity) as well as the oxidation of L-dihydroxyphenylalanine to L-dopaquino-ne (oxidase activity). The L-dopaquinone will be oxidized to form melanin, which has a protective effect against injury caused by ultraviolet radiation. It has been suggested that PPO also plays an important role in melanoma cells [1]. In insects, PPO is considered to be involved not only in melanin formation, but also in sclerotization of cuticles, wound healing, and defense reactions [2].

Funastu and Inaba [3, 4] studied the fluctuation of PPO activity in the process of metamorphosis from the larvae to pupae of the housefly *Musca domestica* L. The most prominent change in housefly PPO activity occurred at the stage of pupation. Specifically, almost the highest PPO activity observed in homogenates of the final instar larvae disappeared suddenly in the homogenates of pre-pupae [3].

However, the disappearance of housefly PPO activity in pre-pupae does not imply vanishing of the enzyme, since pre-pupae homogenate exhibited PPO activity upon addition of an anionic detergent, such as sodium dodecyl sulfate or an extract from aged pupae [4]. These observations suggest that PPO exists in an inactive, latent form in the pre-pupae and that there exists an endogenous activator of the latent enzyme in aged pupae.

The unique roles played by phenol oxidase (PO) in insect physiology and biochemistry certainly demand serious study on this enzyme. By taking advantage of the fact that PO is present in the inactive proenzyme form, some scientists have successfully purified and characterized pro-phenol oxidase. The PPOs from housefly larva [5] and pupa [6] have been purified and their physicochemical characteristics have been determined. Chase et al. [7] reported the purification, characterization, and molecular cloning of pro-phenol oxidases from larval hemolymph of *Sarcophaga bullata*. The enzyme molecular weight was estimated as from 85,000 to 100,000. The activated enzyme exhibited marked thermal instability, and the proenzyme could be specifically activated by cetylpyridinium chloride (CPC). Two pro-phenol oxidase

Abbreviations: PPO) polyphenol oxidase; PO) phenol oxidase; PCR) polymerase chain reaction; L-DOPA) L-3,4-dihydroxyphenylalanine; NBS) N-bromosuccinimide; DTNB) 5,5′-dithiobis(2-nitrobenzoic acid); PGO) phenylglyoxal; CPC) cetylpyridinium chloride.

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genes were PCR (polymerase chain reaction)-amplified and cloned from the sarcophagid larvae by the same research group [7]; the clones encoded polypeptides of 685 and 691 amino acids, contained two distinct copper binding regions, and lacked the signal peptide sequence. In this paper we describe the enzymatic characterization and functional groups of the PPO from pupae of blowfly, which is believed to be involved in the sclerotization and melanization of the blowfly during its life.

MATERIALS AND METHODS

The PPO was prepared from blowfly pupae according to the method of Hall et al. [8], by ammonium sulfate fractionation. The crude preparation was further chromatographed on DEAE-cellulose (DE-32) and subjected to gel filtration through Sephadex G-100. The final preparation was homogeneous on polyacrylamide gel electrophoresis, and the specific activity was determined to be 770 U/mg.

The blowfly pupae were purchased from Carolina Biological Supply Co. (USA); L-3,4-dihydroxyphenylalanine (L-DOPA), N-bromosuccinimide (NBS), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and phenylglyoxal (PGO) were the products of Sigma (USA); cetylpyridinium chloride (CPC) was purchased from Aldrich (USA). DEAE-cellulose was from Whatman (UK); Sephadex G-100 was a Pharmacia (Sweden) product. All other reagents were local products of analytical grade.

Enzyme concentration was determined by the Bradford method with bovine serum albumin as standard. PPO activity assay was performed at 30°C by monitoring the absorbance at 475 nm accompanying the oxidation of the substrate (L-DOPA) (ε = 3700 $M^{-1}\cdot cm^{-1}$) [9]. The reaction system (3 ml) contained 1 mM L-DOPA, 50 mM sodium phosphate (pH 6.8), and 30 μM CPC. One unit (U) of enzymatic activity was defined as the amount of enzyme catalyzing the formation of 1 μmol dopachrome per 1 min from L-DOPA at 30°C. Absorption was recorded using a Beckman UV-650 spectrophotometer. The kinetic and inhibition constants were obtained by the method previously described [10, 11].

RESULTS AND DISCUSSION

Optimum pH and temperature. The effects of pH and temperature on the enzyme activity for the oxidation of L-DOPA were determined. The results indicated that the optimum pH and temperature of the enzyme were 6.5 and 40°C, respectively. The enzyme activity decreased rapidly when the pH value was higher or lower than the optimum pH. PPO from housefly aged pupae was reported to be most active at 18°C [4], and the optimum temperature was 45°C for PPO from shrimp [12]. Phenol oxidases are not extremely heat stable, and are, in most cases, partially or totally destroyed after short exposures to temperatures from 70°C upward [13].

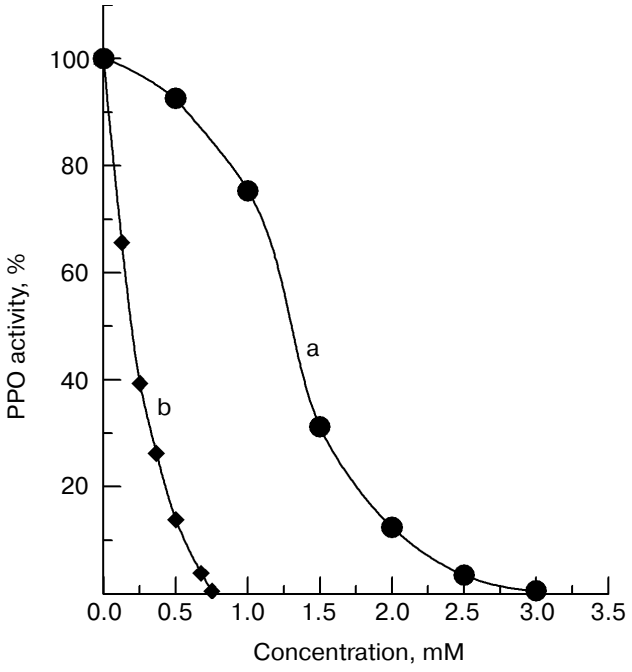

Fig. 1. Effects of DTNB (a) and NBS (b) on polyphenol oxidase activity. The enzyme (0.95 mg/ml) treated with different concentrations of DTNB in 0.1 M Tris-HCl buffer (pH 7.5) at 25°C for 30 min or with different concentrations of NBS in 0.1 M NaAc-HAc buffer (pH 4.5) at 25°C for 5 min. After incubation, 20 μl of reaction mixture was used for activity assay.

Kinetics of the PPO from blowfly pupae. The kinetic behavior of the enzyme in the oxidation of L-DOPA has been studied. Under the conditions used in the present study, the initial reaction rate of the oxidation of L-DOPA by this enzyme followed Michaelis–Menten kinetics. The kinetic parameters have been determined from the Lineweaver–Burk plot. The results showed that K_m and V_{max} were 1.5 ± 0.1 mM and 158 ± 3 μM/min, respectively. The Michaelis constants K_m of PPO from blowfly pupae was lower than that from other animals, such as the housefly pupae with 3.9 mM [6] and the Japanese prawn with 3.45 mM [14].

Chemical modifications of the enzyme. DTNB can specifically modify the sulfhydryl group of cysteine residues under the condition of pH 6.8 [15] and tryptophan residues can be modified by NBS [16]. Figure 1 showed that the activity of the enzyme decreased with increasing the concentrations of DTNB (line (a)) and NBS (line (b)) until the enzyme was completely inactivated. These results indicated that the sulfhydryl and indole groups may be essential to the enzyme activity.

Phenylglyoxal is usually used to modify the guanidyl groups of proteins [17, 18]. The effect of PGO on the

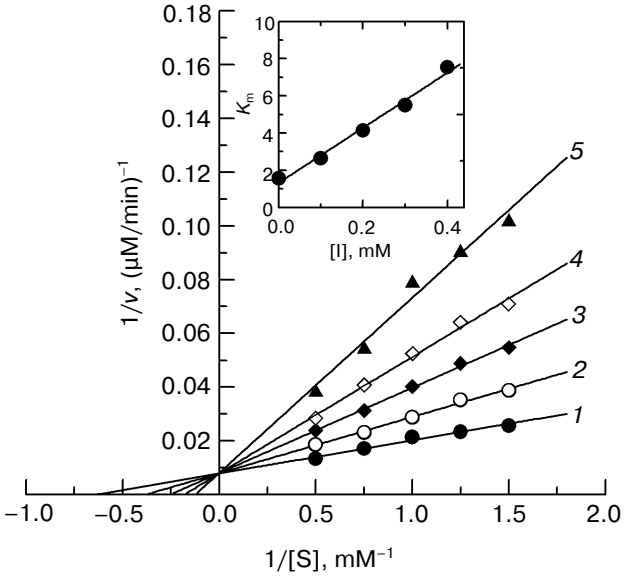

Fig. 2. Lineweaver–Burk plots of the PPO inhibited by quercetin. Concentrations of quercetin for curves *1-5* were 0, 0.1, 0.2, 0.3, and 0.4 mM, respectively. The inset represents the plot of apparent Michaelis constant ($K_{m\ app}$) versus the concentration of quercetin. The finial concentration of the enzyme was 6.5 μg/ml.

enzyme was also studied in 0.1 M Tris-HCl buffer (pH 8.0) at 25°C for 30 min with different concentrations of PGO (from 0.5 to 5.0 mM). The result showed that the enzyme activity remained unchanged, indicating that the guanidyl groups are not essential to the enzyme activity.

Inhibitory effect of quercetin on the enzyme activity. Under the condition employed in the present investigation, the oxidation reaction of L-DOPA by the enzyme followed Michaelis–Menten kinetics (Fig. 2). Quercetin was a competitive inhibitor, as increasing the quercetin concentration resulted in a family of lines with a common intercept on the $1/v$ axis. The equilibrium constant for inhibitor binding with free enzyme, K_I = 88 μM, was obtained from a plot of the apparent Michaelis–Menten constant ($K_{m\ app}$) versus the concentration of quercetin (Fig. 2, inset). From these results, the IC_{50} was estimated to be 0.20 ± 0.06 mM. The inhibition of the enzyme by quercetin was reversible (data not shown). The same result has been reported for mushroom tyrosinase [19]. Presumably, the effect of quercetin comes from its ability to chelate copper in the active center of the enzyme.

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