

Banana Polyphenoloxidase. Preparation and Properties^{1, 2}

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Griffiths (13) has presented evidence indicating that the browning reactions of banana fruit result from the enzymic oxidation of dopamine (3,4 dihydroxyphenylethylamine) by polyphenoloxidase. Although dopamine does occur in various fruits and vegetables (23), it has not previously been implicated as an important substrate in browning reactions. The present investigation was undertaken to develop methods for preparation of banana polyphenoloxidase (PPO)³ and to determine some of its properties in comparison with phenol oxidases from other sources.

Materials and Methods

Materials. The chemicals used and their suppliers were as follows: Dopamine · HCl; D,L-arterenol · HCl; L(-) arterenol bitartrate hydrate; chlorogenic acid; tyramine · HCl; California Corporation for Biochemical Research. 3,4-dihydroxyphenylalanine (DOPA); Nutritional Biochemicals Corp. L(-)tyrosine; Pfanstiehl Laboratories, Inc. Catechol; *o*-cresol; Fisher Scientific Co. *p*-Cresol (redistilled); 8-hydroxyquinoline; L(+)-cysteine · HCl; Matheson, Coleman, and Bell. DEAE-cellulose (Type 20, 0.82 meq/g); Schleicher & Schuell Co.

Assay Procedures. The standard assay for banana PPO was adapted from the tyrosine assay of Fox and Burnett (10). The increase in optical density at 470 m μ after mixing enzyme and substrate was followed at 25° in a DK-2 spectrophotometer equipped with a time drive. The reaction mixture, in a final volume of 3 ml, contained 0.033 M potassium phosphate, pH 7.0; 5×10^{-3} M dopamine and sufficient enzyme to cause an increase in optical density of 0.02 to 0.1 per minute. Rates were calculated from the initial slope of the curve and were proportional ($\pm 10\%$) to enzyme concentration.

Some extracts were assayed indirectly (8) by following spectrophotometrically at 265 m μ the rate of disappearance of ascorbic acid at 25° in a reaction mixture containing 0.05 M potassium phosphate, pH

7.0; 10^{-5} M EDTA; 4.2×10^{-5} M ascorbic acid; 5×10^{-4} M dopamine and enzyme in a final volume of 3 ml. The reaction rate was proportional to enzyme concentration when the decrease in optical density was between 0.02 and 0.2 per minute. All extracts assayed by this method were free of interfering ascorbic acid oxidase.

PPO was also assayed manometrically by standard Warburg technique at 30°. Reaction mixtures are described for the individual experiments.

One unit of PPO activity is expressed wherever possible as that amount of enzyme which will catalyze the transformation of 1 μ mole of substrate per minute under the conditions of the assay. Michaelis constants were obtained from Lineweaver-Burk plots (16).

The protein content of the detergent extracts was estimated from nitrogen determinations (1) on the extract after a 4 hour dialysis against 400 volumes of 0.02 M potassium phosphate, pH 7.0 in a stirring dialyzer. Removal of nonprotein nitrogen was essentially complete under these conditions, but little or no PPO (or detergent) was lost. Protein in the fractions from DEAE cellulose columns was estimated by the method of Waddell (25). Egg albumen (MW = 40,000) and gamma globulin (Bovine Fraction II, MW=150,000) gave identical standard curves. This technique could not be used on the detergent extracts as the detergent interfered.

The spectrometric technique of Mason (17) was used to study the reaction mechanism. A series of 1 μ mole samples of dopamine in 10 ml of 0.05 M potassium phosphate, pH 6.0 were oxidized by 50 mg of Ag₂O with shaking for periods varying between 1.5 and 20 minutes. The solutions were filtered quickly, and the resulting spectra were compared with those of the enzymic oxidation products. D, L-DOPA was oxidized under similar conditions to check the procedure.

Dopamine β -oxidase activity was assayed by the technique of Smith and Kirshner (21). A one ml aliquot of diluted (1:50) detergent extract was incubated with 1 μ mole of dopamine at 37° for 1 hour. The reaction was stopped with 1 ml of 10% trichloroacetic acid and the mixture centrifuged at low speed to sediment protein. A 1.2 ml aliquot was extracted 3 times with ether and then assayed fluorimetrically (9) to determine arterenol (Beckman DK-2 spectrophotometer, No. 22850 fluorescence attachment, 515 m μ).

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² A preliminary report of this work has appeared in *Plant Physiol.* 36, Suppl. xxxviii, 1961.

³ Terminology used in this paper: Phenol oxidase, generic term to include all enzymes which catalyze the oxidation of phenols; tyrosinase, enzyme which catalyzes the oxidation of both mono- and diphenols; polyphenoloxidase, enzyme which catalyzes the oxidation of ortho dihydric phenols only.

Results

Extraction of Soluble PPO. Ripe banana fruits (*Musa acuminata*, var. Hort. Gros Michel) were the source of the enzyme. All operations were conducted at 0 to 5° unless otherwise specified.

Initial experiments were carried out with buffered homogenates (1–2 g of tissue in 20 ml of 0.1 M potassium phosphate, pH 6 or 7) of banana pulp or peel tissue. These homogenates catalyzed the aerobic oxidation of dopamine and other diphenols, resulting in the production of brown pigments. All activity was lost on boiling these homogenates and the browning reactions were inhibited by ascorbic acid, sodium hydrosulfite, cysteine, diethyldithiocarbamate, and potassium ethyl xanthate. Pulp tissue was chosen for further study as the pulp homogenates were essentially colorless whereas peel homogenates darkened rapidly during the preparative procedure.

The browning reactions in pulp homogenates invariably took place on the surface of the tissue fragments. When the homogenates were centrifuged at $500 \times g$ for 10 minutes, 80 to 100 % of the PPO activity was sedimented with these fragments. Any remaining activity was in the supernatant fraction after further centrifugation at $20,000 \times g$ for 15 minutes. These results suggested that the PPO was adsorbed on or structurally associated with the cell wall. Releasing the enzyme into solution was accomplished by extraction of the pulp with buffered detergent solution. Pulp (2 g) was homogenized in a 50 ml Duall Tissue Grinder (Kontes Glass Company) in 18 ml of a 1 % detergent solution buffered at pH 7.0 with 0.02 M or 0.1 M potassium phosphate. The non-ionic, polyoxyethylated detergents known as Cutscum (Fisher Scientific Company) and Igepal CO-630 (General Aniline and Film Company) were equally effective in rendering the enzyme soluble. The PPO activity was in the supernatant solution after centrifugation at $20,000 \times g$ for ca. 15 minutes. These detergent extracts were appropriately diluted (usually 1:50) with 0.02 M potassium phosphate, pH 7.0 for use in the assay procedures.

This procedure not only solubilized banana PPO, but also increased the activity by a factor of 1.5 to 2.5 (table I). Cotzias et al. (5) reported similar results in their studies on monoamine oxidase. Detergent at 1 % approached the minimum concentration required for efficient extraction of banana polyphenoloxidase from pulp tissue. Little or no additional activity was extracted at 5 % while extracts with 0.1 % detergent contained about one-half of the activity at 1 %. Extracts of comparable pulp samples with 1 % detergent solution had essentially identical activity. However, extraction of the sedimented debris with fresh aliquots of detergent yielded about 15 % additional activity.

The detergent extracts were clear to slightly cloudy, faint yellow-brown in color and free of significant endogenous activity. These readily prepared extracts were used for all subsequent studies on

Table I

Polyphenoloxidase Activity of Banana Pulp Extracts.

Experiment number	QO ₂ * (μl O ₂ per mg dry wt per hr)	
	Homogenate	Detergent extract
1	105	154
2	168	403

* Assay conditions: Experiment No. 1, 0.067 M dopamine; 0.067 M potassium phosphate, pH 6.0; 0.5 ml enzyme (undiluted detergent extract). Experiment No. 2, 0.017 M dopamine; 0.067 M potassium phosphate, pH 6.0; 0.2 ml enzyme. Final volume (including 0.2 ml 5 % KOH in center well) was 3.0 ml in each case.

the properties and purification of banana PPO, unless otherwise noted.

Dopamine can also be oxidized directly or indirectly by laccase, monoamine oxidase, cytochrome oxidase, or peroxidase. The banana fruit undoubtedly contains some or all of these enzymes. However, the detergent extracts were free of significant amounts of these enzymes as determined by appropriate assay (12, 20) and by failure of the extracts to oxidize monophenols, β-phenylethylamine, *p*-phenylenediamine or ascorbic acid (3, 6, 22).

Purification of Banana PPO. The enzyme was freed of excess detergent by precipitation from the undiluted detergent extract at –8° with 1.6 volumes of acetone. Significant amounts of detergent were not precipitated from these extracts until 1.7 volumes of acetone had been added. The precipitated material was collected by centrifugation ($3500 \times g$, 20 minutes, –8°) and then stirred up in a volume of 0.02 M potassium phosphate, pH 7.0, equal to one-half the original volume of detergent extract. After standing overnight the PPO had redissolved and the mixture was centrifuged at $20,000 \times g$ for 10 minutes to remove the inactive residue. The supernatant fluid was added to a 1.0 cm \times 10 cm column of DEAE cellulose which had been previously washed and equilibrated with 0.04 M phosphate, pH 8.0 by the procedure of Keller et al. (15). The flow rate of the column (1 ml/min; 1 lb pressure) was usually reduced as the sample entered the column and addition was stopped when pressures of 8 lbs/sq in were required to maintain a flow rate of about 8 ml/hr. The column was then washed with 100 ml of 0.04 M phosphate, pH 8.0 at 8 ml/hr, followed by 0.08 M phosphate, pH 8.0 at the same flow rate, according to the procedure of Frieden and Ottesen (11). Some preparations did not reduce the flow rate appreciably and these were eluted at 15 ml/hr under about 1 lb pressure. Fractions of 2.5 to 3.5 ml were collected and assayed for protein content and PPO activity (standard assay).

Figure 1 shows the results of a typical chromatographic separation. A 10 to 12 fold purification of the PPO from detergent extracts was obtained in

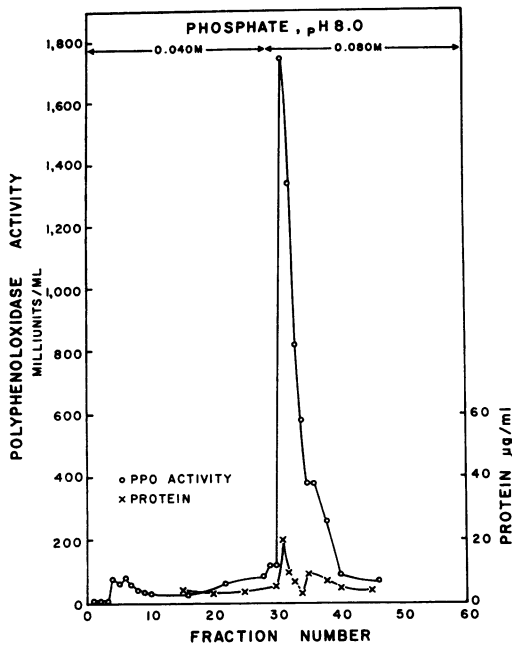


FIG. 1. Chromatography of banana polyphenoloxidase on DEAE cellulose. See text for details.

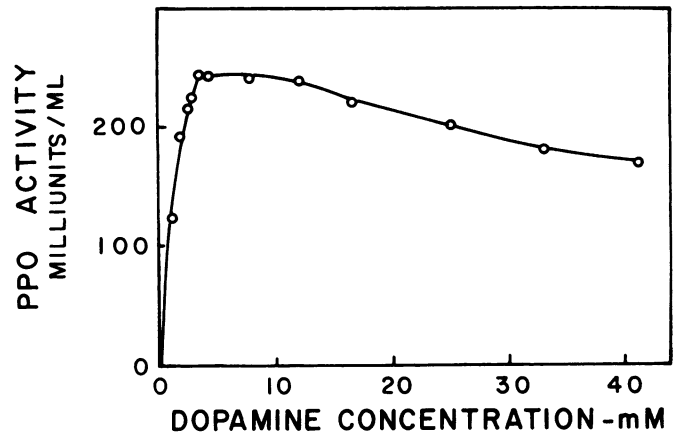


FIG. 2. Effect of dopamine concentration on activity of banana polyphenoloxidase. Standard assay.

the fractions of highest specific activity from the DEAE column (table II). These pooled fractions, hereafter referred to as purified extract, were used in some of the substrate studies described below.

Substrate Studies. The substrate specificity of detergent extracts and purified extracts was identical. A variety of *o*-dihydric phenols were oxidized, dopamine having the lowest K_m value among the substrates tested (table III). Monophenols such as tyrosine, tyramine, *o*-cresol or *p*-cresol were not oxidized. No reaction was observed for up to 1 hour after mixing the enzyme with the monophenols at 0.002 M to 0.02 M. Addition of catechol or dopamine in an attempt to eliminate any induction period had no effect on the oxidation of tyrosine.

The K_m for dopamine determined with the standard spectrometric assay was considerably smaller

than that determined from the oxygen uptake (table III), presumably because the spectrometric assay specifically measures the initial rate of formation of the first pigmented product, while the O_2 uptake arises from a series of oxidations involved in melanogenesis. Similar differences in the K_m of chlorogenic acid as a substrate for the polyphenol oxidase of *Solidago virgaurea* leaves have been reported by Björkman and Holmgren (2).

The enzyme was saturated by dopamine concentrations higher than 3 mM, but inhibition set in above 12 mM (fig 2).

pH Optimum. Banana PPO had a pH optimum of 7.0 when catalyzing the oxidation of dopamine (fig 3). Negligible autoxidation of this substrate occurred in the standard assay at pH 7 or below. The values at pH 7.5 and 8.0 in figure 3 were cor-

Table II
Purification of Polyphenoloxidase from Banana Fruit

Fraction	Volume ml	Activity* units/ml	Total activity units	Protein mg/ml	Specific activity units/mg protein	Recovery %
Detergent extract	40	4.7	188	0.48	9.8	100
Acetone ppt	19	8.9	169	90
DEAE eluate	9.5**	2.3	21.8	0.02	115	12***

* Standard assay at pH 7.0.

** Pooled fractions of highest specific activity from DEAE separation.

*** Actual recovery in this experiment, where only 10.5 ml of the acetone ppt was separated on DEAE column. Normally the final yield of high specific activity enzyme will be nearer 20 %.

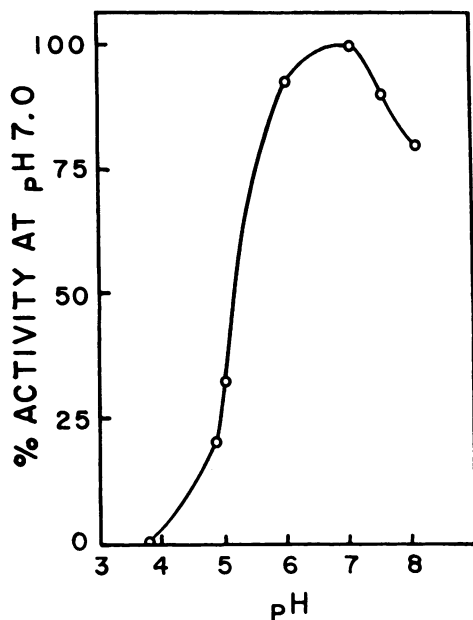
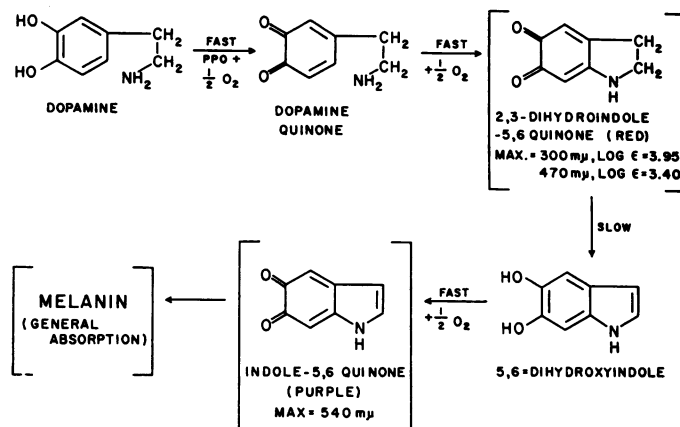


FIG. 3. Effect of pH on activity of banana polyphenoloxidase. Standard assay, dopamine substrate. pH 6 to 8, phosphate buffer; pH 3.8 to 6, citrate buffer.

FIG. 4. Proposed reaction mechanism for the oxidation of dopamine by banana polyphenoloxidase. Spectrochemical evidence was obtained for the presence of the bracketed compounds only. The remaining intermediates are assumed by analogy with DOPA oxidation (18), and not all the probable intermediates are shown.



rected for 9 and 20% autoxidation, respectively.

Variation in the final phosphate buffer concentration from 0.01 M to 0.067 M or substitution of citrate buffer for phosphate buffer at pH 6 had no effect on the activity.

Stability of Enzyme. The undiluted detergent extracts usually retained at least 90% of their original dopamine oxidase activity for 2 weeks and at

least 70% for a month at 2 to 5°. The diluted detergent extracts and the purified extracts were somewhat less stable, sometimes losing 30% of their activity in 1 week at 2 to 5°. Detergent extracts could usually be frozen and thawed without effect, but occasional preparations lost significant activity after such treatment. At room temperature the enzyme in detergent extracts was stable for at least 24 hours. In one case, it retained 60% of its activity after 1 week at room temperature.

Reaction Mechanism. The first spectroscopically observable product of the PPO catalyzed oxidation of dopamine was a red pigment with absorbancy peaks at 300 m μ and 470 m μ . If the oxidation was continued beyond about 15 minutes, a purple pigment ($\lambda_{\text{max}} = 540 \text{ m}\mu$) was produced and finally a grey to black precipitate appeared. Oxidation of dopamine for increasing periods of time with silver oxide, as described by Mason (17), yielded the same pigments. Log ϵ for the red pigment was 3.95 at 300 m μ and 3.40 at 470 m μ . Log ϵ for the purple pigment could not be determined because of its transitory nature. Essentially identical spectral data were also obtained for the enzymic and chemical oxidation products from DOPA, in agreement with Mason (17).

Based on this spectrochemical evidence, the reaction mechanism shown in figure 4 is proposed for the enzymic oxidation of dopamine by banana PPO. It is analogous to that proposed by Mason (18) for DOPA oxidation.

Table III

Michaelis Constant of Substrates for Banana Polyphenoloxidase

Substrate	K_m^* M	Assay
Dopamine	6.3×10^{-4}	Standard
D,L-arterenol	2.4×10^{-3}	"
L-arterenol	3.6×10^{-3}	"
D,L-DOPA	4.4×10^{-2}	"
L-DOPA	6.6×10^{-2}	"
D-DOPA	3.0×10^{-2}	"
Catechol	2.6×10^{-3}	Indirect
Dopamine	4×10^{-3}	Manometric**
Chlorogenic acid	3×10^{-2}	"

* Average of at least 2 determinations with either detergent extracts or purified extracts. No significant differences were noted for the 2 different preparations.

** Assay conditions: 0.025 M potassium phosphate, pH 7.0; 0.2 ml enzyme (undiluted detergent extract); 0.2 ml 5% KOH (center well); substrate and water to the final volume of 2 ml.

One other possible mechanism for the production of melanins in banana extracts was considered. Smith and Kirshner (21) reported the presence of an enzyme (termed dopamine β -oxidase by Senoh et al. (19)) in bananas which converts dopamine to arterenol. Since arterenol is a substrate for banana PPO (table III), this compound could be the actual substrate for melanin formation in banana extracts. Furthermore, the initial oxidation products of arterenol would have spectra essentially identical to those derived from dopamine.

The diluted detergent extracts contained sufficient dopamine β -oxidase to convert up to 20% of the original dopamine to arterenol in 1 hour, when assayed by the technique of Smith and Kirshner (21). However, because of the marked difference in the K_m of the 2 compounds, it is unlikely that arterenol plays any significant role in the *in vitro* system. For example, if we make the unlikely assumption that 10% of the dopamine in the standard polyphenoloxidase assay was instantaneously converted to arterenol by the dopamine β -oxidase present, the concentration of arterenol would be only 5×10^{-4} M. No significant pigment formation resulted when this concentration of arterenol was incubated with banana PPO. These observations do not preclude the possibility that both mechanisms may occur in living tissue.

Discussion

Phenol oxidases are copper proteins of wide occurrence in nature which catalyze the aerobic oxidation of certain phenolic substrates to quinones which are autoxidized to dark brown pigments generally known as melanins. For discussion purposes, these enzymes are assumed to be single enzymes with broad specificity, although there is some evidence for the presence of more than one phenol oxidase in certain tissues (4).

The phenol oxidase extracted with detergent from the pulp of the ripe banana fruit is by substrate specificity a polyphenoloxidase and is similar in this respect to the enzymes of sweet potato (7, 24), tobacco (4), tea leaf (24), and *Solidago virgaurea* leaf (2). In contrast, the enzymes from most other sources such as potato, mushroom, and several mammalian tissues are tyrosinases (or true tyrosinases) which catalyze the oxidation of both mono- and diphenols (24).

Yasunobu (24) has recently reviewed the substrate specificity of a number of phenol oxidases from various sources, both plant and animal. He concluded that these enzymes catalyze the oxidation of a wide variety of substrates, but that each individual enzyme tends to catalyze the oxidation of one particular phenol (or a particular type of phenolic compound) more readily than others. The results reported here show that banana PPO follows this general pattern, with dopamine being the most readily oxidized substrate.

It is significant that dopamine is the most reactive substrate for the banana enzyme since this compound occurs at exceptionally high levels (1–2 mg/g fr wt) in the peel of the banana fruit (E. H. Buckley, unpublished) and has been shown to be the only significant substrate in the browning reactions of this fruit (13). To the author's knowledge, this is the clearest link between the substrate specificity of a phenol oxidase from a particular plant material and the demonstrated substrate for browning reactions in that material. Further study of this unique relationship could help to answer some of the problems concerning the synthesis, localization, and role of phenol oxidases in plant tissue.

Dopamine is also the primary substrate for PPO in the roots and rhizome of the banana plant (M. E. Mace, unpublished). Although it occurs in a variety of other plant tissues (23), it has seldom been tested as a substrate for phenol oxidase. Dopamine has recently been shown to be the most reactive substrate ($K_m = 2 \times 10^{-4}$ M, spectral assay at 465 m μ) for the purified PPO from blowfly larvae, where it appears to be a key intermediate in sclerotization (14). This compound could prove to be important in the browning reactions of many plant and animal tissues.

There are 2 other noteworthy points regarding the substrate specificity of banana PPO. First, although dopamine and L-DOPA differ chemically only by the presence of a carboxyl group on the sidechain, the K_m values for these 2 compounds differ by a factor of 100. Second, in contrast to other phenol oxidases, D-DOPA appears to be a more reactive substrate than L-DOPA. Additional study will be required to assess the significance of these observations.

Summary

Polyphenoloxidase has been shown to occur in the pulp and peel of the banana fruit. It can be readily extracted from the pulp and rendered soluble in buffered detergent solution. These preparations were essentially colorless, and had little or no endogenous activity. The enzyme was further purified by acetone precipitation and chromatography on DEAE-cellulose.

The soluble enzyme catalyzed the oxidation of a variety of diphenolic substrates. K_m values for a number of these were determined and they indicate that dopamine is most readily oxidized. Banana polyphenoloxidase in detergent extracts was relatively stable and had optimum activity at pH 7 with dopamine as the substrate. The mechanism of dopamine oxidation appears to be analogous to that for the oxidation of DOPA by tyrosinase.

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