Purification and Characterization of Polyphenol Oxidase from Banana (*Musa sapientum* L.) Pulp

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Polyphenol oxidase (EC 1.10.3.1, PPO) in the pulp of banana (*Musa sapientum* L.) was purified to 636-fold with a recovery of 3.0%, using dopamine as substrate. The purified enzyme exhibited a clear single band on polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE. The molecular weight of the enzyme was estimated to be about 41000 and 42000 by gel filtration and SDS−PAGE, respectively. The enzyme quickly oxidized dopamine, and its K_m value for dopamine was 2.8 mM. The optimum pH was at 6.5, and the enzyme activity was stable in the range of pH 5−11 at 5 °C for 48 h. The enzyme had an optimum temperature of 30 °C and was stable even after a heat treatment at 70 °C for 30 min. The enzyme activity was completely inhibited by L-ascorbic acid, cysteine, sodium diethyldithiocarbamate, and potassium cyanide. Under a low buffer capacity, the enzyme was also strongly inhibited by citric acid and acetic acid at 10 mM.

Keywords: *Banana (Musa sapientum L.) pulp; polyphenol oxidase; dopamine oxidase; purification; characterization*

INTRODUCTION

Undesirable browning in damaged tissues of fresh fruits and vegetables is induced by enzymatic oxidation of polyphenols, mainly by polyphenol oxidase (EC 1.10.3.1, PPO). To prevent such enzymatic browning, which results in a decreased marketability, many PPOs have been characterized (Luh and Phithakpool, 1972; Kahn, 1976, 1977; Wissemann and Lee, 1981; Tono et al., 1986; Fujita and Tono, 1988; Fujita et al., 1991; Murata et al., 1992; Chilaka et al., 1993; Sojo et al., 1998). However, most of these studies have been conducted using crude and/or partially purified PPO except for the studies on such fruits and vegetables as Japanese pear (Tono et al., 1986), lettuce (Fujita et al., 1991), and apple (Murata et al. 1992).

To characterize the enzyme properties, pyrocatechol, 4-methylcatechol, and chlorogenic acid have been widely used as major substrates (Luh and Phithakpool, 1972; Benjamin and Montgomery, 1973; Kahn, 1976; Fujita and Tono 1988, Fujita et al., 1991). In the eggplant and lettuce, the enzymatic browning is mainly caused by the enzymatic oxidation of chlorogenic acid which exists in large quantities (Fujita and Tono, 1988; Fujita et al., 1991). In the peel and pulp of banana (*Musa sapientum* L.), dopamine was detected in large quantity (Riggin et al., 1976; Tono et al., 1999), and it was strongly oxidized by the crude and/or partially purified PPO (Palmer, 1963; Galeazzi et al., 1981a). These results indicate that the enzymatic browning in the banana pulp section appears to be due to the oxidation of dopamine by the endogeneous PPO. However, little is known about the PPO oxidizing dopamine. In the present study, banana pulp PPO was purified, and some properties of the purified enzyme were investigated.

MATERIALS AND METHODS

Materials. Banana fruits in the yellow-green stage of ripening, which were imported by Dole Co. from Philippines, were purchased from a local market in Saga City. DEAE-Toyopearl 650M, Butyl-Toyopearl 650M, and Toyopearl HW55-Superfine (HW55-S) were purchased from Toso Co., Tokyo; hydroxyapatite was obtained from Biorad Co., Biorad, CA. Glucoamylase was purchased from Nagase Biochemical Co., Osaka, Japan. Other reagents were obtained from Wako Pure Chemical Co., Osaka, and Katayama Chemical Co., Osaka, Japan.

Measurement of PPO Activity. PPO activity was determined by the colorimetric method. The reaction mixture consisted of 1.0 mL of 0.02 M aqueous solution of dopamine, 3.9 mL of 0.1 M sodium phosphate−0.1 M potassium phosphate buffer (0.1 M PB, pH 7), and 0.1 mL of the enzyme solution. After incubation at 30 °C for 5 min, the absorbance of the mixture at 420 nm was measured. One unit of the enzyme activity was defined as an increase in absorbance of the mixture at 420 nm (ΔA_{420}) of 0.1 per minute and per milliliter of enzyme solution (1 cm light path).

Purification of the Enzyme. All steps were carried out at about 5 °C. Banana pulp (7.8 kg) was homogenized with 1300 mL of 0.1 M PB (pH 7) containing 5.0 g glucoamylase, and the homogenate was hydrolyzed by glucoamylase at 37 °C for 3 h. After the filtration of the hydrolyzed homogenate through cotton cloth, the filtrate was centrifuged at 10300 g for 20 min, and solid ammonium sulfate was added to the supernatant to 80% saturation. The protein precipitate, collected by centrifugation (20 min at 10300 g), dissolved in a small volume of 0.06 M PB (pH 7) and dialyzed against the same buffer for 36 h, with at least four changes of the dialyzing media. The dialyzed solution was added to a DEAE-Toyopearl 650M column (2.7 × 12.5 cm) equilibrated with the 0.06 M PB (pH 7) and eluted with the same buffer. PPO fractions, which were not adsorbed on the column, were pooled, brought to a 1 M ammonium sulfate concentration, applied to Butyl-

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10.1021/jf991037+ CCC: $19.00
Published on Web 06/09/2000

Table 1. Stepwise Purification Results of PPO of Banana Pulp

purification step	volume (mL)	total activity (unit)	total protein (mg)	specific activity (unit/mg protein)	purification (fold)	recovery (%)
crude extract	6350	388620	18732.5	20.7	1.0	100.0
80% $(NH_4)_2SO_4$ saturation (crude enzyme)	298	239592	2972.6	80.6	3.9	61.7
DEAE-Toyopearl 650M	324	158120	822.0	192.5	9.3	40.7
Butyl-Toyopearl 650M	370	100317	360.2	278.5	13.4	25.8
Super Q-Toyopearl 650M	50	32400	5.6	5785.7	279.5	8.3
Hydroxyapatite	20	15360	1.4	10971.4	530.0	4.0
Toyopearl HW 55-S	94	11844	0.9	13160.0	635.7	3.0

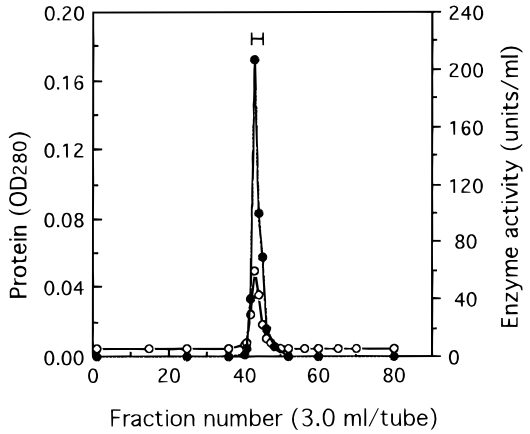

Figure 1. Elution pattern of the enzyme on Toyopearl HW 55S. ○ Protein, ● PPO activity, − fractions pooled.

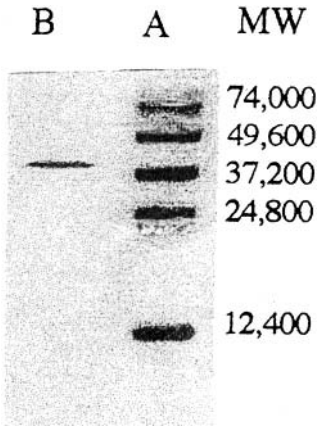

Figure 2. SDS−PAGE of the purified enzyme. A, marker protein; B, purified enzyme; MW, molecular weight.

Toyopearl 650M column (1.6 × 10.5 cm) equilibrated with 0.06 M PB (pH 7) containing 1 M ammonium sulfate, and eluted with a linear gradient of ammonium sulfate (1 M to 0 M ammonium sulfate in 0.06 M PB, pH 7). The enzyme-active fractions were collected and dialyzed against 0.02 M PB (pH 7). The dialyzed solution was added to Super Q-Toyopearl 650M column (2.2 × 9.5 cm) equilibrated with 0.02 M PB (pH 7) and eluted with a linear gradient of sodium chloride (0 to 0.2 M NaCl in 0.02 M PB, pH 7). The enzyme active fractions were pooled and dialyzed against 0.01 M PB (pH 6.8). The dialyzed enzyme solution was added to a hydroxyapatite column (2.2 × 11.0 cm) equilibrated with 0.01 M PB (pH 6.8) and eluted with a linear gradient of 0.01 M to 0.2 M PB (pH 6.8). The enzyme fractions were collected, finally applied to Toyopearl HW 55-S column (1.6 × 89.0 cm) equilibrated with 0.1 M PB (pH 7), and eluted with the same buffer. The PPO-active fractions were pooled as purified PPO for the following characterization.

Determination of Protein. Protein was determined by the method of Hartree (1972), using a bovine serum albumin (Fraction V, Katayama Chemical Co., Osaka) as standard. In chromatography, protein was expressed with absorbance at 280 nm.

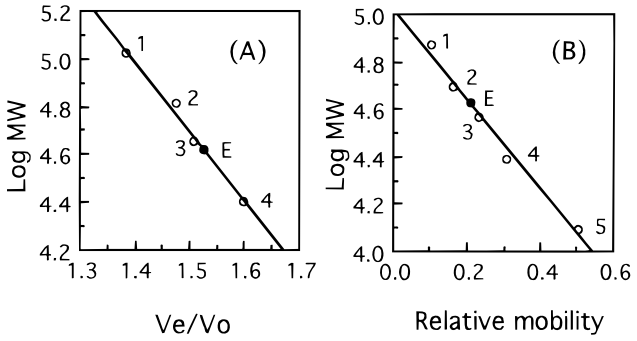

Figure 3. Molecular weight estimation of the enzyme by gel filtration on Toyopearl HW 55F (A) and SDS−PAGE (B). Figure 3 (A): V_o, void volume of the column; V_e, elution volume of the substance; MW, molecular weight. 1, γ -globulin; 2, bovine serum albumin; 3, egg albumin; 4, chymotrypsinogen A, 5; cytochrome *c*; E, purified enzyme. Figure 3 (B): MW, molecular weight. Cytochrome *c*: 1, hexamer (74000); 2, tetramer (49600); 3, trimer (37200); 4, dimer (24800); 5, monomer (12400); E, purified enzyme.

Poly Acrylamide Gel Electrophoresis (PAGE). Electrophoresis of the purified enzyme was carried out following the method of Davis (1964), using 7.5% polyacryl amide gel at pH 9.4.

Molecular Weight Estimation. Molecular weight of the purified enzyme was estimated by gel filtration and SDS−polyacrylamide gel electrophoresis (SDS−PAGE).

Gel filtration was done following the method of Andrews (1965), using cytochrome *c* (MW 12500), chymotrypsinogen A (25000), egg albumin (45000), bovine serum albumin (65000), and γ -globulin (125000) as marker proteins.

SDS−PAGE was carried out as described by Weber and Osborn (1969), using an SDS marker protein kit (Oriental Yeast Co., Tokyo) as standard.

RESULTS AND DISCUSSION

Purification of the Enzyme. In this experiment, the viscous polysaccharide in banana pulp was first hydrolyzed by glucoamylase, because the polysaccharide interfered with filtration and anion-exchange chromatography in the purification procedures. Figure 1 shows the typical elution pattern of the enzyme on the final Toyopearl HW 55-S gel filtration. The enzyme activity was shown in one peak and the peak fractions from several columns were pooled as the purified PPO. The typical result of stepwise purification of the enzyme is given in Table 1. Finally, PPO was purified up to 636-fold with a recovery rate of 3.0% as compared with crude extract.

Some Properties of the Purified Enzyme. The purified enzyme produced a single band on PAGE and SDS−PAGE (Figure 2). As shown in Figure 3, the molecular weight was estimated to be about 41000 by gel filtration and 42000 by SDS−PAGE. These results indicate that the purified enzyme may be a monomer. Galeazzi et al. (1981a) already reported the dimer PPO in Nanica banana pulp, the molecular weight of which

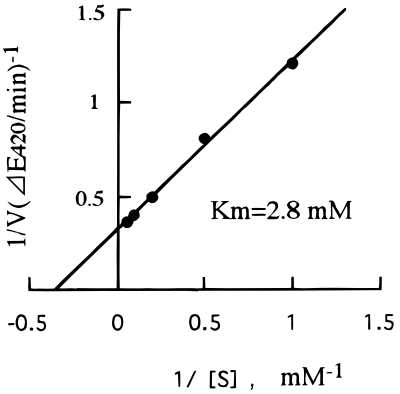

Figure 4. Lineweaver−Burk plots of dopamine oxidation by the enzyme. *V*, rate of oxidation; *S*, substrate concentration.

Table 2. Substrate Specificity of PPO of Banana Pulp

substrate	relative activity (%)
dopamine	100.0
catechol	54.0
D-catechin	35.6
chlorogenic acid	24.5
epicatechin	22.7
DL-Dopa	12.3
pyrogallol	5.5
caffeic acid	2.0
gallic acid	0
phloroglucinol	0
resorcinol	0

was estimated to be about 62000 by sucrose gradient ultracentrifugation. The difference in the molecular weights may be due to the different banana varieties used. The molecular weight is smaller than that of chlorogenic acid oxidase from apple (Murata et al., 1992) and of PPO from Japanese pear (Tono et al., 1986), which were estimated to be about 65000 and 56000, respectively.

As shown in Table 2, the enzyme quickly oxidized dopamine. The oxidation rate of catechol and D-catechin by the purified PPO is about one-half and one-third that of dopamine, respectively. The enzyme had very weak and/or no activity toward such 1,2,3-trihydroxybenzenes as pyrogallol and gallic acid. The results indicate that dopamine is the main substrate for PPO in banana pulp, as demonstrated by Griffiths (1959) and Palmer (1963). Therefore, the purified banana PPO can be considered as a dopamine oxidase. Such substrate specificity of the banana enzyme differs from PPO of apple (Murata et al., 1992), Japanese pear (Tono et al., 1986), eggplant (Fujita and Tono., 1988), and lettuce (Fujita et al., 1991), which markedly oxidized chlorogenic acid. As shown in Figure 4, the K_m value of the purified enzyme for dopamine oxidation is 2.8 mM, and the value is larger than 0.17 mM of the enzyme purified from the inner part of the banana pulp of Nanica var. (Galeazzi et al., 1981a).

Figure 5 shows the effect of pH on the activity and stability of the enzyme. The enzyme optimum pH for dopamine was 6.5, and was similar to the optimum pH of PPOs in kiwi fruit (Park and Luh, 1985), royal ann cherry (Benjamin and Montgomery, 1973), and guava (Augustin et al., 1985). However, it was different from PPOs (pH 4) of apple (Murata et al., 1992) and eggplant (Fujita and Tono, 1988). More than 90% of the original activity was retained between pH 5 and 11 after 48 h incubation at pH 3 to 11. The pH stability is similar to cabbage PPO (Fujita et al., 1995, 1997), but is different

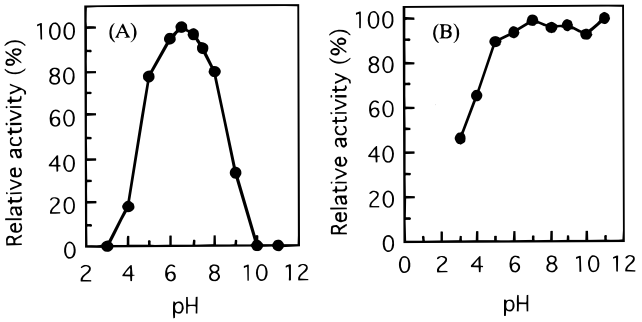

Figure 5. Effect of pH on the activity (A) and stability (B) of the enzyme.

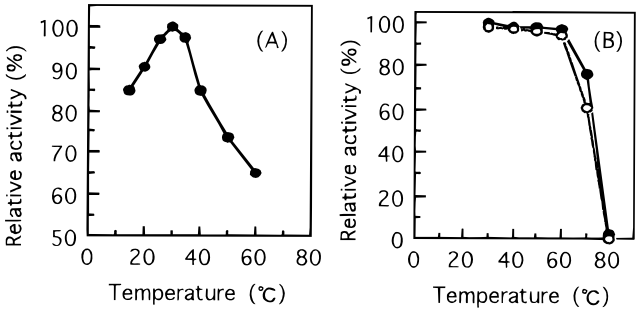

Figure 6. Effect of temperature on the activity (A) and stability (B) of the enzyme. Figure 6(B): ●, 10 min: ○, 30 min.

Table 3. Effect of Various Compounds on PPO Activity of Banana Pulp

compound	relative activity (%) 1mM[a]	10 mM[a]
none	100.0	100.0
sodium diethyldithiocarbamate	0	0
KCN	19.3	0
EDTA	99.5	98.4
NaF	98.6	98.6
$MnCl_2$	95.6	94.2
$BaCl_2$	96.2	99.7
$CuSO_4$	97.2	99.7
$ZnSO_4$	97.3	91.5
NaCl	98.5	98.5
L-ascorbic acid	0	0
cysteine	0	0
citric acid	99.6 (86.1[b])	92.2 (0[b])
acetic acid	99.4 (95.5[b])	95.8 (38.6[b])

[a] Final concentration of various compounds. [b] Using 0.01 M PB (pH 7) instead of 0.1 M PB (pH 7).

from PPOs of apple (Murata et al., 1992), lettuce (Fujita et al., 1991), Japanese pear (Tono et al., 1986), and eggplant (Fujita and Tono, 1988).

As shown in Figure 6, the purified enzyme had an optimum temperature of 30 °C and was relatively stable at high temperatures; about 80% of the PPO activity remained after a heat treatment at 70 °C for 10 min. Relatively high thermal stabilities were found in the PPOs in kiwi fruit (Park and Luh, 1985), mango (Park et al., 1980), Satsuma mandarin (Fujita and Tono, 1979), and head lettuce (Fujita et al., 1991).

As listed in Table 3, the enzyme activity was markedly inhibited by sodium diethyl-dithiocarbamate and potassium cyanide; however, EDTA and metal ions (Cu^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+}) were poor inhibitors of the enzyme at 10 mM. The enzyme activity was also completely inhibited by L-ascorbic acid and cysteine at 1 mM. Similar inhibitory effects of these compounds on banana pulp PPO were also demonstrated (Galeazzi et

al., 1981b; Sojo et al., 1998). Acetic acid, citric acid, and sodium chloride weakly inhibited the banana pulp PPO activity at 10 mM at standard pH condition of this experiment (0.1 M PB, pH 7). However, under low buffer capacity (using 0.01 M PB, pH 7), PPO activity was markedly inhibited by citric acid and acetic acid at 10 mM, in which the pH of the reaction mixture was 3.1 and 4.4, respectively. Similar inhibitory effects of acetic acid and citric acid were found in the browning of head lettuce (Castaner et al. 1996). These results suggest that L-ascorbic acid, cysteine, citric acid, and acetic acid are good inhibitors of enzymatic browning of banana pulp.

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Received for review September 17, 1999. Revised manuscript received April 4, 2000. Accepted April 25, 2000.

JF991037+