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

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Polyphenol oxidase (EC 1.10.3.1, *o*-diphenol: oxygen oxidoreductase, PPO) of banana (*Musa sapientum* L.) peel was partially purified about 460-fold with a recovery of 2.2% using dopamine as substrate. The enzyme showed a single peak on Toyopearl HW55-S chromatography. However, two bands were detected by staining with Coomassie brilliant blue on PAGE: one was very clear, and the other was faint. Molecular weight for purified PPO was estimated to be about 41 000 by gel filtration. The enzyme quickly oxidized dopamine, and its *Km* value (Michaelis constant) for dopamine was 3.9 mM. Optimum pH was 6.5 and the PPO activity was quite stable in the range of pH 5−11 for 48 h. The enzyme had an optimum temperature at 30 °C and was stable up to 60 °C after heat treatment for 30 min. The enzyme activity was strongly inhibited by sodium diethyldithiocarbamate, potassium cyanide, L-ascorbic acid, and cysteine at 1 mM. 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.) peel; enzymatic browning; polyphenol oxidase; purification; characterization*

INTRODUCTION

Undesirable browning of damaged tissue is known to be catalyzed by polyphenol oxidase (EC 1.10.3.1, *o*-diphenol: oxygen oxidoreductase, PPO) during storage and processing of fresh fruits and vegetables. PPO has been widely studied in many fruits and vegetables to determine how to prevent the browning which results in the loss of their marketability (*1*−*12*). The banana (*Musa sapientum* L.) pulp PPO has been characterized (*13*−*17*), because a remarkable browning is found in the section of the pulp. The PPO strongly oxidized dopamine, and was found in a large quantity in the pulp (*18*, *19*). Such browning was also found in the peel containing a large amount of dopamine (*18*, *19*). However, purification and characterization of peel PPO has not yet been done, and little is known about the enzyme properties. The objective of this study was to compare the properties of peel PPO to those of pulp PPO (*17*). In this study, PPO was highly purified from banana peel using dopamine as substrate, and some properties of the enzyme were determined.

MATERIALS AND METHODS

Materials. Banana fruits (Dole Company, Philippines) in the yellow-green stage of ripening 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, Japan. Hydroxyapatite was obtained from Biorad Co., Hercules, 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.

Assay of PPO Activity. PPO activity was determined by a 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 by Shimadzu MPS-2000 spectrophotometer (Shimadzu Co., Kyoto, Japan). One unit of the enzyme activity was defined as a change in absorbance of the mixture at 420 nm (ΔA_{420}) of 0.1 per min and per ml enzyme solution (1 cm light path).

Purification of the Enzyme. All steps were carried out at 5 °C. Banana peel (9.7 kg) was homogenized in 6 000 mL of 0.1 M PB (pH 7) containing 0.1% glucoamylase, 2% Tween 80, and 5 mM ascorbic acid. The resulting homogenate was hydrolyzed by glucoamylase at 37 °C for 3 h. After filtration of the hydrolyzed homogenate through cotton cloth, the filtrate was centrifuged at 10 300×*g* for 20 min, and solid ammonium sulfate was added to the supernatant to 80% saturation. The precipitate was collected by centrifugation for 20 min at 10 300×*g*, dissolved in a small volume of 0.06 M PB (pH 7), and dialyzed against the same buffer for 36 h, with four changes of the dialyzing medium. The dialyzed solution was added to a DEAE-Toyopearl 650M column (2.8 × 13.5 cm) equilibrated with 0.06 M PB (pH 7), and eluted with a linear gradient of sodium chloride (0 to 0.2 M NaCl in 0.06 M PB, pH 7). Enzyme-active fractions were pooled and brought to contain 1 M ammonium sulfate and applied to a Butyl-Toyopearl 650M column (1.6 × 11.5 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 in 0.06 M PB, pH 7). The enzyme-active fractions were collected and dialyzed overnight against 0.02 M PB (pH 7)**.** The dialyzed solution was added to a Super Q-Toyopearl 650M column (2.2 × 12.5 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). Enzyme-active fractions were pooled and dialyzed overnight against 0.01 M PB (pH 6.8). The dialyzed enzyme solution was added to a hydroxyapatite column (2.2 × 11.5 cm) equilibrated with 0.01 M PB (pH 6.8), and eluted with a linear gradient of 0.01 M to 0.3 M PB (pH 6.8).

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Table 1. Purification of Polyphenol Oxidase from Bananna Peel[a]

purification step	volume (ml)	total activity (units)	total protein (mg)	specific activity (unit/mg protein)	purification (fold)	recovery (%)
crude extract	12200	775920	34430.0	22.5	1.0	100.0
80% $(NH_4)_2SO_4$ saturation (crude enzyme)	732	394694	8857.2	44.6	2.0	50.9
DEAE-Toyopearl 650M	191	116476	144.3	807.2	35.9	15.0
Butyl-Toyopearl 650M	381	64465	19.5	3305.9	146.9	8.3
SuperQ-Toyopearl 650M	37	39960	5.0	7992.0	355.2	5.2
hydroxyapatite	84	25402	2.7	9408.1	418.1	3.3
Toyopearl HW55−S	73	16819	1.6	10511.9	467.2	2.2

[a] This experiment is one trial with triplication and is representative of the data seen with four additional trials.

The enzyme fractions were collected and concentrated to about one-fourth the original volume by a Butyl-Toyopearl 650M column (1.5 × 4.0 cm), which was equilibrated with 1 M ammonium sulfate in 0.1 M PB (pH 7), and then stepwise eluted with 0.1 M PB (pH 7). The enzyme-active fractions were collected and applied to a 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 (*20*), using a bovine serum albumin (Fraction V, Katayama) as standard. For chromatography, protein was expressed as the absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis (PAGE). Electrophoresis of the purified enzyme was carried out according to the method of Davis (*21*), using 7.5% polyacrylamide gel at pH 9.4.

Molecular Weight Estimation. Molecular weight of the purified enzyme was estimated by gel filtration according to the method of Andrews (*22*), using chymotrypsinogen A (25 000), egg albumin (45 000), bovine serum albumin (65 000), and *r*-globulin (125 000) as marker proteins.

RESULTS AND DISCUSSION

Purification of the Enzyme. Sojo et al. (*23*) reported that the banana pulp PPO existed as a "latent form" which was extracted by buffer containing detergent such as Triton X-114. Recently, we reported that most of the banana peel PPO was also present as a latent form (*24*). In this experiment, a detergent (Tween 80) was used to solubilize the PPO which was bound strongly to the peel tissue and/or tannins (*15, 23*). In addition, viscous polysaccharide in peel, which interfered with filtration and anion-exchange chromatography, was hydrolyzed by glucoamylase to help release the bound PPO (*17*). On the final Toyopearl HW 55-S gel filtration, the enzyme activity showed a single peak and the peak fractions were pooled as partially purified PPO (Figure 1). However, two bands were detected in the enzyme solution by staining with Coomassie brilliant blue on PAGE: one was very clear, and the other was faint (Figure 2). The typical results of stepwise purification results for the enzyme are given in Table 1. In these purification procedures, a Butyl-Toyopearl column (1.5−4.0 cm) had been used before gel-filtration to concentrate the enzyme, and all of the enzyme activity was recovered. The data were not included in the Table. Finally, PPO was purified about 470-fold with a recovery rate of 2.2% as compared with crude extract. These results suggested that the banana peel PPO was highly purified, and the purified enzyme was used for all remaining experiments.

Characterization of the Enzyme. The molecular weight of the enzyme was estimated (by gel filtration) to be about 41 000 (Figure 3). This weight is almost the same as that of banana pulp PPO (*17*), but is different

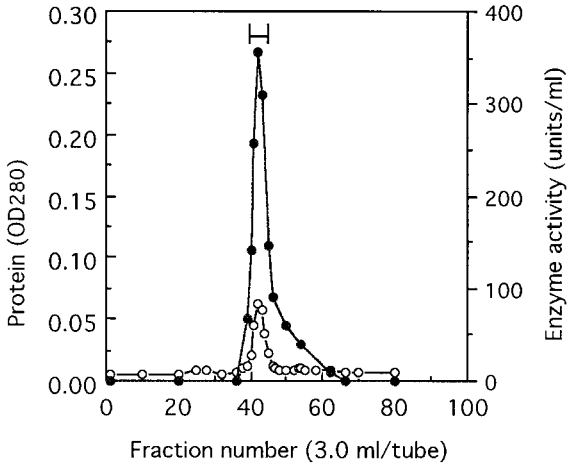

Figure 1. Elution pattern of the enzyme on Toyopearl HW 55-S. ○ Protein, ● PPO activity, — fractions pooled.

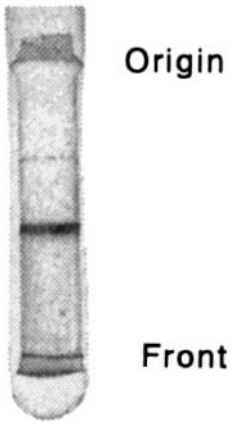

Figure 2. Polyacrylamide gel electrophoresis of the partially purified enzyme.

from that of pulp PPO from the other banana species (*Musa cavendishii* L, var. Nanica) being estimated to be about 62 000 by sucrose gradient ultra centrifugation (*15*). The difference in the molecular weight may be due to the different banana species used.

As shown in Table 2, the enzyme strongly oxidized dopamine. However, the oxidation rates of catechol, catechin, and chlorogenic acid by the peel PPO were below one-third that of dopamine. The enzyme had no activity toward resorcinol and phloroglucinol. Similar substrate specificity was also found in banana pulp PPO (*13, 14, 17*). These results indicate that the purified banana-peel PPO can be considered as a dopamine oxidase like the pulp PPO. Therefore, the substrate specificity for banana PPOs is different from those of apple (*10*), Japanese pear (*5*), eggplant (*6*), and lettuce (*7*) PPOs; the latter oxidized chlorogenic acid markedly. The Michaelis constant (*Km* value) of peel PPO for

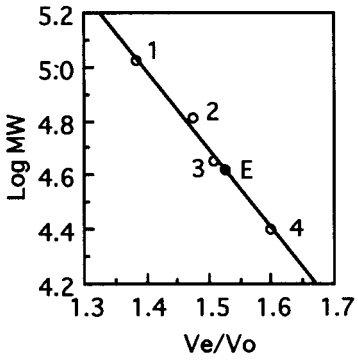

Figure 3. Molecular weight estimation of the enzyme by gel filtration on Toyopearl HW 55-S. *Vo*, void volume of the column; *Ve*, elution volume of the substance; MW, molecular weight. 1, γ-globulin; 2, bovine serum albumin; 3, egg albumin; 4, chymotrypsinogen A; E, purified enzyme.

Table 2. Substrate Specificity of the Enzyme[a]

substrate	relative activity (%)
dopamine	100
catechol	34.0
D-catechin	11.5
epicatechin	9.3
chlorogenic acid	5.3
DL-dopa	8.0
pyrogallol	1.4
caffeic acid	0.7
gallic acid	0
phloroglucinol	0
resorcinol	0

[a] This experiment is one trial with triplication and the table is representative of the data seen with four additional trials.

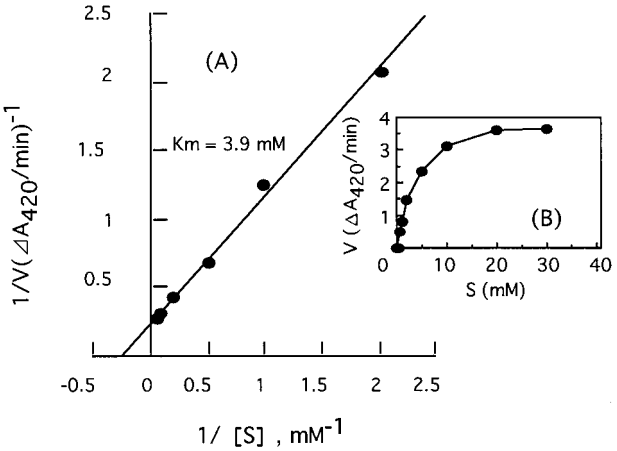

Figure 4. Lineweaver-Burk plots (A) and Michaelis−Menten curve (B) of dopamine oxidation by the enzyme. V, rate of oxidation; S, substrate concentration.

dopamine oxidation was 3.9 mM (Figure 4), which is similar to that of pulp PPO (2.7 mM, *17*), but was higher than that for PPO from the inner part of banana pulp of the variety Nanica (0.17 mM, *16*). Banana peel contains a large amount of dopamine (*18, 19*), and dopamine is strongly oxidized by peel PPO. These results suggested that the browning of banana peel is mainly due to the enzymatic oxidation of dopamine.

As shown in Figure 5A, the optimum pH of the banana peel PPO for dopamine oxidation was 6.5. The value is similar to that of PPOs in banana pulp (*17*), kiwi fruit (*25*), royal ann cherry (*2*), guava (*26*), and Satsuma mandarin (*27*). However, it was different from the PPOs of Japanese pear (*5*) and apple (*10*). As shown in Figure 5B, the enzyme activity is quite stable in the

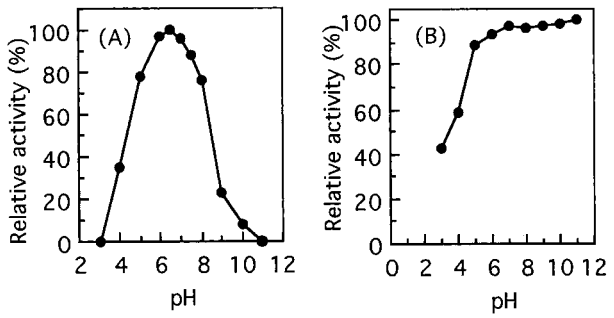

Figure 5. Effect of pH on the activity (A) and stability (B) of the enzyme. Final concentration of the dopamine was 4 mM. This effect is one trial with triplication and is representative of the effects seen with four additional trials.

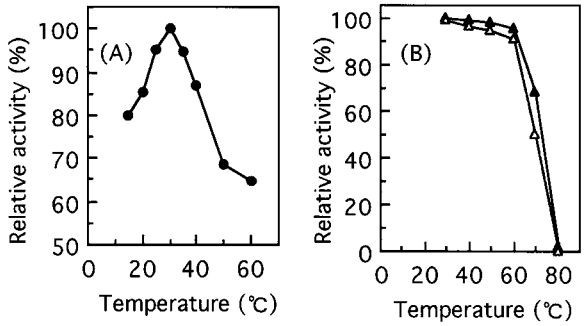

Figure 6. Effect of temperature on the activity (A) and stability (B) of the enzyme. ▲, 10 min; △, 30 min. Final concentration of dopamine was 4 mM. This effect is one trial with triplication and is representative of the effects seen with four additional trials.

Table 3. Effect of Various Compounds on Polyphenol Oxidase[a]

compound	relative activity (%)	
	1 mM[b]	10 mM[b]
none	100.0	100.0
sodium diethyldithiocarbamate	0	0
KCN	11.0	0
EDTA	92.7	89.9
NaF	99.2	99.6
$MnCl_2$	99.6	99.6
$BaCl_2$	98.9	98.0
$CuSo_4$	93.1	89.5
$ZnSO_4$	99.6	89.2
NaCl	98.9	97.9
L-asorbic acid	0	0
cysteine	0	0
citric acid	99.3 (90.4[c])	97.9 (0[c])
acetic acid	99.3 (96.3[c])	97.2 (48.1[c])

[a] This effect is one trial with triplication and is representative of the effects seen with four additional trials. [b] Final concentrations of various compounds. [c] Using 0.01 M PB (pH 7) instead of 0.1 M PB (pH 7).

range of pH 5−11. The pH stability is similar to those of banana pulp PPO (*17*) and cabbage PPO (*8, 9*), but is different from the PPOs of apple (*10*) and eggplant (*6*).

Figure 6 shows the effect of temperature on the activity and stability of the enzyme. The optimum temperature of the enzyme was 30 °C. The enzyme was relatively stable at high temperatures: about 90% of the activity remained after heat treatment at 60 °C for 30 min. Similar high thermal stability has been found for PPOs in banana pulp (*17*), kiwi fruit (*25*), mango (*28*), lettuce (*7*), and cabbage (*8, 9*).

Table 3 shows that the enzyme activity is markedly inhibited by sodium diethyl-dithiocarbamate and potas-

sium cyanide, and poorly inhibited by EDTA and metal ions (Na^+, Cu^{2+}, Mn^{2+}, and Zn^{2+}) at 10 mM. The activity was also strongly inhibited by L-ascorbic acid and cysteine at 1 mM. Similar effects of these compounds were found in the PPOs of banana pulp (*15*, *17*). Acetic acid, citric acid, and sodium chloride weakly inhibited the PPO activity at 10 mM at standard pH condition (0.1 M PB, pH 7). However, under a 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 these acids on PPOs under a low buffer capacity were also reported in head lettuce (*29*) and banana pulp (*17*). These results suggest that cysteine, L-ascorbic acid, citric acid, and acetic acid are able to be used as good inhibitors of enzymatic browning in banana peel.

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