Note

Purification and Characterization of Polyphenol Oxidase from Edible Yam (*Dioscorea opposita* Thunb.)

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Soluble polyphenol oxidase of edible yam (*Dioscorea opposita* Thunb.) was purified approximately 203-fold with a recovery rate of 15% by ammonium sulfate fractionation, ion exchange chromatography, hydrophobic chromatography and gel filtration using dopamine as a substrate. The purified enzyme appeared as a single band on native PAGE and SDS-PAGE. The molecular weight of the enzyme was estimated to be approximately 42kDa and 44kDa using gel filtration and SDS-PAGE, respectively. The purified enzyme quickly oxidized dopamine. The apparent K_m value (Michaelis constant) of the enzyme was 1.5 mM for dopamine (pH 7.0, 30°C). The optimum pH was 7.0 for dopamine oxidase. In the pH range from 6 to 10, the activity was quite stable at 5°C for 22 h. The optimum temperature of enzyme activity was 25–30°C. The activity was stable up to 50°C after heat treatment for 20 min. The browning reaction by the enzyme was completely inhibited by 1 mM L-ascorbic acid, which reduced o-quinone to dopamine. The reaction was also completely inhibited by 1 mM L-cysteine, which is a known quinone coupler. About 35% inhibition of edible yam PPO was observed using citric acid and acetic acid at 10 mM in 0.1 M citrate/ 0.2 M sodium phosphate buffer (pH 7). In consideration of the observed results, L-ascorbic acid, L-cysteine, acetic acid, and citric acid are expected to be used as effective inhibitors of enzymatic browning in edible yam.

Keywords: polyphenol oxidase, edible yam, dopamine oxidase, purification

Introduction

Edible yam (*Dioscorea opposita* Thunb.) is a popular vegetable in Japan. During the processing of this vegetable, undesirable browning occurs in the damaged tissue, primarily due to oxidation of polyphenols by polyphenol oxidase (EC 1.10.3.1; o-diphenol: oxygen oxidoreductase, PPO). Enzymatic browning also occurs in other fruits and vegetables, resulting in lowered marketability. For the prevention of such browning, many investigations have been conducted in order to characterize PPOs in fruits and vegetables (Anosike and Ayaebene, 1981; Ikediobi and Obasuyi, 1982; Tono *et al.*, 1986; Fujita and Tono, 1988; Fujita *et al.*, 1991; Murata *et al.*, 1992; Fujita *et al.*, 1995; Fujita *et al.*, 1997; Ding *et al.*, 1998; Yang *et al.*, 2000; Nkya *et al.*, 2003; Kihara *et al.*, 2005). To characterize the enzyme properties, pyrocatechol, 4-methyl catechol and chlorogenic acid have been widely used as major substrates (Fujita and Tono, 1988; Fujita *et al.*, 1991; Murata *et al.*, 1992; Ding *et al.*, 1998; Nkya *et al.*, 2003; Kihara *et al.*, 2005). Characterization of yam PPO was also performed using catechol as

a substrate (Anosike and Ayaebene, 1981; Ikediobi and Obasuyi, 1982; Asemota *et al.*, 1992; Omidigi and Okpuzor, 1996). However, in edible yam and konjak tubes, large quantities of dopamine were detected, which were primarily oxidized by their crude and/or partially purified PPO (Tono, 1970; Tono, 1971; Tono *et al.*, 1974; Tono *et al.*, 1999). These results indicate that enzymatic browning in yam is mainly caused by the oxidation of dopamine by endogeneous PPO (dopamine oxidase); however, little is known about the dopamine oxidase in yam tuber. In our laboratory, we isolated and characterized PPOs, which primarily oxidize dopamine, from banana pulp (Yang *et al.*, 2000). Although the crude PPO from edible yam oxidized dopamine more quickly than other phenols, to date, the characteristics of this enzyme have not been investigated in detail. In the present study, edible yam PPO was purified using dopamine as a substrate, and the properties of the purified enzyme were investigated.

Materials and Method

Materials Edible yam (*D. opposita*) was purchased at local markets in Saga city, Japan. DEAE-Toyopearl 650 M, Butyl Toyopearl 650 M and Toyopearl HW 55-Super-

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fine (HW55-S) were obtained from Toso Co., Tokyo. All other reagents used were purchased from Wako Pure Chemical Co., Osaka, and Katayama Chemical Co., Osaka, Japan.

Assay of enzyme activity PPO activity was measured using a colorimetric method (Fujita and Tono, 1988; Fujita *et al.*, 1991; Yang *et al.*, 2000) in a reaction mixture containing 0.5 mL of 10 mM aqueous solution of various phenols (see Table 2), 4 mL of 0.1 M citrate/0.2 M sodium phosphate buffer (McIlvaine buffer, pH 7) and 0.5 mL of enzyme solution. After 5 min incubation of the mixture at 30°C, the increase in absorbance at 420 nm (ΔA_{420}) was measured using a Shimadzu MPS-2000 spectrophotometer. One unit of enzyme activity was defined as an increase of absorbance of 0.1 per minute and per milliliter of enzyme solution (1 cm light path) at 420 nm.

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

Enzyme purification All purification steps were carried out at 5°C. Edible yam tubers (fresh weight 1.7 kg) were homogenized with 1000 mL of 0.1 M potassium phosphate/0.1 M sodium phosphate buffer (0.1 M phosphate buffer, PB, pH 7) containing 5% *n*-hexadecylpyridinium chloride and 0.5% L-ascorbic acid. After filtration of the homogenate through cotton cloth, the filtrate was centrifuged at $10300 \times g$ for 20 min, and the supernatant was brought to 80% ammonium sulfate saturation. After stirring overnight, the protein precipitate was collected by centrifugation at $10300 \times g$ for 20 min, dissolved in a small volume of 0.01 M PB (pH 7), and then dialyzed against the same buffer for 36 h during which the dialyzed media was changed four times. The dialyzed solution (crude enzyme) was applied to a DEAE-Toyopearl column (3.2×14.5 cm) equilibrated with 0.01 M PB (pH 7) and the enzyme fractions, which were not adsorbed on the column, were collected. The enzyme fractions containing 2.0 M ammonium sulfate were applied to a Butyl-Toyopearl column (1.6×26 cm), equilibrated with 0.01 M PB containing 2.0 M ammonium sulfate, and eluted with a linear gradient of ammonium sulfate (2.0 M to 0 M in 0.01 M PB, pH 7). The enzyme active fractions were pooled and dialyzed against 0.01 M phosphate buffer (pH 7) with the dialyzed media changed four times. The dialyzed enzyme solution was applied to a Toyopearl HW 55-S (1.6×89 cm) column equilibrated with 0.1 M phosphate buffer (pH 7) and eluted with the same buffer. The PPO active fractions were pooled and used for enzyme characterization.

Polyacrylamide gel electrophoresis (PAGE) Electrophoresis of the purified enzyme was carried out according to the method of Davis (1964), using 7.5% polyacrylamide gel at pH 9.0.

Molecular weight determination The molecular weight of the purified enzyme was estimated by gel filtration and SDS-PAGE. Gel filtration was conducted using a Toyopearl HW 55-S column (1.6×89.0 cm), which was equilibrated and eluted with 0.1 M phosphate buffer (pH 7),

according to the method of Andrews (1965). Chymotrypsinogen A (25 kDa), egg albumin (45 kDa), bovine serum albumin (65 kDa), and γ -globulin (150 kDa) were used as marker proteins at a flow rate of 20 mL per hour.

SDS-PAGE was carried out according to the method described by Laemmli (1970), using SDS-marker protein kit (Bio Rad Co., Hercules CA, USA).

Results and Discussion

Enzyme purification In this experiment, the mucous polysaccharide in the edible yam extract was initially removed by the addition of *n*-hexadecylpyridinium chloride (Araki *et al.*, 1995), because the polysaccharide interfered with the cotton cloth filtration and anion exchange chromatography in the purification procedures. The enzyme was purified from the homogenates of edible yam by ammonium sulfate fractionation, and chromatography on DEAE-Toyopearl 650M, Butyl Toyopearl 650 M, and Toyopearl HW 55-S gel filtration. The purified enzyme showed a single band on native PAGE and SDS-PAGE (Fig. 1). These results suggest that edible yam PPO was purified to a homogeneous state. The typical result for the stepwise purification of the enzyme is given in Table 1. Finally, the enzyme was purified approximately 203-fold with a recovery rate of 15% when compared with the crude extract.

Some properties of edible yam PPO As shown in Fig. 2, the molecular weight of the native enzyme was estimated to be approximately 42 kDa by gel filtration, and a subunit molecular weight of 44 kDa by SDS-PAGE. These results may indicate that the purified PPO is a monomer. The molecular weight of the purified enzyme was much smaller than that of white yam (*D. rotundata*) PPO (MW 107 kDa), which quickly oxidized catechol (Ikediobi and Obasuyi, 1982). However, the molecular weight of our

Fig. 1. SDS-PAGE (A) and PAGE (B) of the purified PPO of edible yam.

(A); PM, protein marker; E, purified enzyme; MW, molecular weight.

(B), Enzyme, purified enzyme.

Table 1. Purification summary of polyphenol oxidase of edible yam.

Procedure	Total protein (mg)	Total activity (KU)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude extract	13603	205	15.1	1.0	100.0
80% (NH ₄) ₂ SO ₄ saturation	1118	74	66.6	4.4	36.1
DEAE-Toyopearl 650M	1125	56	50.0	3.3	27.3
Butyl-Toyopearl 650M	26.3	39	1480	98.0	19.0
Toyopearl HW-55S	9.8	30	3068	203.0	14.6

Each value is the mean of three trials.

Fig. 2. Estimation of molecular weight (MW) of purified PPO of edible yam by gel filtration on SDS-PAGE (A) and Toyopearl HW 55S (B).

(A): 1, myosin (214 kDa); 2, β -galactosidase (118 kDa); 3, bovine serum albumin (92 kDa); 4, ovalbumin (52.2 kDa); 5, carbonic anhydrase (35.7 kDa); 6, soy bean trypsin inhibitor (28.9 kDa); 7, lysozyme (20.8 kDa); E, purified enzyme. (B): V_o , void volume of the column; V_e , elution volume of the substance; 1, γ -globulin (150 kDa); 2, bovine serum albumin (65 kDa); 3, egg albumin (45 kDa); 4, chymotrypsinogen A (25 kDa); E, purified enzyme.

Table 2. Substrate specificity of edible yam PPO.

Substrate	Relative activity (%)
Dopamine	100
Catechol	35
Epicatechin	20
Chlorogenic acid	6.5
DL-Dopa	6.5
Pyrogallol	15
Gallic acid	0
Phloroglucinol	0
Resorcinol	0

Reactions were carried out in McIlvaine buffer (pH 7) at 30°C. Each value is the mean of three trials.

D. opposita purified PPO was similar to that of the banana pulp dopamine oxidase (Yang *et al.*, 2000), which has an estimated molecular weight of approximately 41 kDa and 42 kDa by gel filtration and SDS-PAGE, respectively. The molecular weight of *D. opposita* PPO was smaller than that of the PPO of Japanese pears (MW 56 kDa) (Tono *et al.*, 1986), head lettuce (MW 56 kDa) (Fujita *et al.*, 1991), and

apple (MW 65 kDa) (Murata *et al.*, 1992), which quickly oxidized chlorogenic acid.

Table 2 shows substrate specificity of the purified enzyme. The purified enzyme quickly oxidized dopamine. The oxidation rate for pyrocatechol and epicatechin by the PPO was low compared to that of dopamine. The enzyme had very weak or no activity towards such tri-

hydroxybenzenes as phloroglucinol and gallic acid. In contrast, purified white yam (*D. rotundata*) PPO quickly oxidized catechol and chlorogenic acid (Ikediobi and Obasuyi, 1982). The different substrate specificity between *D. opposita* and *D. rotundata* may due to the difference in the species and/or cultivar of yam. Similar substrate specificity of *D. opposita* PPO was observed for the banana pulp PPO (Yang *et al.*, 2000). Such substrate specificity of PPOs in *D. opposita* and banana also differs from the PPO of Japanese pear (Tono *et al.*, 1986), eggplant (Fujita and Tono, 1988), lettuce (Fujita *et al.*, 1991), apple (Murata *et al.*, 1992), and garland chrysanthemum (Nka *et al.*, 2003), which quickly oxidized chlorogenic acid. In edible yam, dopamine was detected in large quantities (Tono, 1970; Tono, 1971; Tono *et al.*, 1999). These results suggest that enzymatic browning in yam is primarily caused by the oxidation of dopamine by PPO. Therefore, the purified *D. opposita* PPO can be considered a dopamine oxidase. The K_m value of the enzyme was 1.5 mM, measured from Lineweaver-Burk plots using dopamine as a substrate (pH 7.0, 30°C). This value is similar to that of banana pulp dopamine oxidase (Yang *et al.*, 2000), which has a value of 2.8 mM.

Other properties of the purified enzyme are listed in Table 3. The purified PPO showed a single but broad pH optimum curve between pH 6 to 8 with maximum activity at pH 7. This value is similar to the optimum pH of PPOs

in kiwi fruit (Park and Luh, 1985), guava (Augustin *et al.*, 1985), banana pulp (Yang *et al.*, 2000), and another species of yam tuber (Anosike and Ayaebene, 1981; Ikediobi and Obasuyi, 1982). However, it differed from PPOs of eggplant (Fujita and Tono 1988), apple (Murata *et al.*, 1992), and garland chrysanthemum (Nkya *et al.*, 2003), which have an optimum pH of 4. More than 80% of the original activity remained after incubation for 48 h between pH 4 and 10. The pH stability differed slightly from the PPOs of Japanese pear (Tono *et al.* 1986), eggplant (Fujita and Tono, 1988), lettuce (Fujita *et al.*, 1991), apple (Murata *et al.*, 1992), banana pulp (Yang *et al.*, 2000), and garland chrysanthemum (Nkya *et al.*, 2003), for which, more than 90% of the PPOs' original activity remained after incubation for 48 h between pH 4 and 10.

The purified PPO showed a single but broad optimum temperature curve from 20 to 30°C with maximum activity at 30°C. This value is similar to the optimum temperature of the PPO of another species of yam tuber (Ikediobi and Obasuyi, 1982; Omidiji and Okpuzor, 1996). The PPO activity remained unchanged after heat treatment at 50°C for 30 min. These results indicated that the enzyme has relatively high thermal stability. Similar thermal stability has also been reported for the PPOs of another species of yam (Ikediobi and Obasuyi, 1982), kiwi fruit (Park and Luh, 1985), lettuce (Fujita *et al.*, 1991), cabbage (Fujita *et al.*, 1995), banana pulp (Yang *et al.*, 2000), and garland

Table 3. Some properties of edible yam PPO.

1. Optimum pH ¹⁾	7	
2. pH stability ²⁾	4-10	
3. Optimum temperature ³⁾	30 °C	
4. Thermal stability ⁴⁾	50 °C	
5. Effectors ⁵⁾	Relative activity (%)	
Compounds	1 mM ^a	10 mM ^a
None	100.0	100.0
Sodium diethyldithiocarbamate	0.0	0.0
EDTA	91.5	76.9
KCN	1.7	0.5
NaF	95.7	77.5
NaCl	95.7	85.5
BaCl ₂	87.5	—
ZnSO ₄	93.9	—
L-Ascorbic acid	0.0	0.0
L-Cysteine	0.0	0.0
Citric acid	88.4	63.0
Acetic acid	85.5	66.7

1) Reactions were carried out at different pH levels at 30°C.

2) Reactions were carried out in Mcillvaine buffer (pH 7.0) at 30°C.

3) Reactions were carried out in Mcillvaine buffer (pH 7.0).

4) Reactions were carried out in Mcillvaine buffer (pH 7.0) at 30°C.

5) The reactions were carried out in Mcillvaine buffer (pH 7.0) at 30°C. Each value is the mean of three trials. ^a; Final concentration of various compounds —; not determined (solutions were cloudy).

chrysanthemum (Nkya *et al.*, 2003).

The enzyme activity was almost completely inhibited by sodium diethyldithiocarbamate and potassium cyanide at 1 mM. Only 5–13% inhibition of the enzyme reactions was observed following the addition of metal ions (Ba^{2+} , Zn^{2+}), EDTA, and sodium fluoride at 1 mM. The browning reaction of the enzyme was completely inhibited by 1 mM L-ascorbic acid, which reduced o-quinone to dopamine. The reaction was also completely inhibited by 1 mM L-cysteine, which is a known quinone coupler. Similar effects of these compounds have been observed for PPOs of another species of yam (Ikediobi and Obasuyi, 1982), Japanese pear (Tono *et al.* 1986), head lettuce (Fujita *et al.*, 1991), and cabbage (Fujita *et al.*, 1995). For dopamine oxidase in banana pulp (yang *et al.*, 2000), its activity is markedly inhibited by citric acid and acetic acid at 10 mM under relatively low buffer capacities (using 0.01 M PB, pH 7). In contrast, approximately 35% inhibition of edible yam dopamine oxidase was observed for the use of citric acid and acetic acid at 10 mM with relatively high buffer capacity (using McIlvaine buffer, pH 7). Similar effects of citric acid and acetic acid on edible yam dopamine oxidase have been reported for garland chrysanthemum PPO (Nkya *et al.*, 2003).

In conclusion, PPO of edible yam (*D. opposita*) was purified to approximately 203-fold with a recovery rate of 15%, using dopamine as a substrate. The molecular weight and substrate specificities of the purified PPO (dopamine oxidase) differed from those of white yam (*D. rotundata*) PPO (catechol oxidase); however, the effect of pH, temperature, and various compounds on enzyme activity were similar. These findings indicate that the difference in substrate specificity and molecular weight between *D. opposita* and *D. rotundata* was due to the different species and/or cultivar of yam. Because dopamine was detected in large quantities (Tono, 1970; Tono, 1971; Tono *et al.*, 1999) and the PPO quickly oxidized dopamine, the enzymatic browning in yam appears to be primarily due to the oxidation of dopamine by the dopamine oxidase. In consideration of the present results, L-ascorbic acid, L-cysteine, acetic acid, and citric acid are expected to be used as effective inhibitors of enzymatic browning in edible yam.

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