Purification and Properties of Polyphenol Oxidase from Cabbage (*Brassica oleracea* L.)

Shuji Fujita,*,† Nazamid bin Saari,† Mitsuru Maegawa,† Toshisuke Tetsuka,† Nobuyuki Hayashi,† and Tetsuzo Tono‡

Laboratory of Food Science, Faculty of Agriculture, Saga University, Saga 840, Japan, and Faculty of Food Science and Nutrition, Nishikyushu University, Kanzaki 842, Saga, Japan

Polyphenol oxidase (PPO) in cabbage (*Brassica oleracea* L.) was purified using phloroglucinol as substrate. The purified enzyme was found to be of a homogeneous state by PAGE and SDS–PAGE. The molecular weight of the enzyme was estimated to be about 39 000 and 40 000 by gel filtration and SDS–PAGE, respectively. The purified enzyme only oxidized 1,3,5-trihydroxybenzenes such as phloroglucinol (K_m = 6.4 mM) and phloroglucinolcarboxylic acid. The enzyme also had strong peroxidase (POD) activity. The optimal pH values of PPO and POD were 7.6 and 6.4, respectively, and both activities were stable in the pH ranges 6–11 at 5 °C for 20 h. Both activities had very high thermal stability; about 40% of the PPO and about 25% of the POD activities remained after heat treatment at 100 °C for 10 min. Both activities were markedly inhibited by sodium diethyldithiocarbamate and potassium cyanide. $MnCl_2$ markedly activated PPO activity but strongly inhibited POD activity.

Keywords: *Cabbage (Brassica oleracea L.); polyphenol oxidase; phloroglucinol oxidase; characterization*

INTRODUCTION

Polyphenol oxidase (EC 1.10.3.1; PPO) catalyzes the oxidative reaction associated with undesirable browning of damaged tissues in fresh fruits and vegetables. To prevent the browning, which results in decreased marketability of products, the enzyme has been widely studied (Luh and Phithakpool, 1972; Kahn 1976, 1977; Wissemann and Lee, 1981; Augustin et al., 1985; Tono et al., 1986; Fujita and Tono, 1988; Fujita et al., 1991; DaDamio and Thompson, 1992; Murata et al., 1992; Chilaka et al., 1993). This enzyme strongly oxidized *o*-diphenols such as pyrocatechol, 4-methylcatechol, chlorogenic acid, and DL-Dopa. In previous papers, we reported that a new type of PPO, which only oxidized 1,3,5-trihydroxybenzenes such as phloroglucinol and phloroglucinolcarboxylic acid, was found in Satsuma mandarin (Fujita and Tono, 1979, 1980a) and turnip (Fujita and Tono, 1980b) and that the purified enzymes of them also had strong peroxidase (EC 1.11.1.7; POD) activity. The phloroglucinol oxidase (PhO) and POD activities were found in the crude enzyme of cabbage (Nawa et al., 1987); however, the details of the cabbage PPO were not investigated. It is interesting that both PhO and POD activities occur in the same enzyme as well as in Satsuma mandarin and turnip enzymes. In the present study cabbage PPO was purified by chromatography and the PhO activity of the fractions tested by using phloroglucinol as the substrate; some properties of the purified enzyme were investigated.

MATERIALS AND METHODS

Materials. Fresh cabbage (*Brassica oleracea* L.) was purchased from a local market in Saga. DEAE-cellulose (DE23) was obtained from Whatman Co., Maidstone, Kent, England. DEAE-Toyopearl 650M, butyl-Toyopearl 650M, and Toyopearl HW 55F were obtained from Toso Co., Tokyo. CM-Sephadex C-50 was obtained from Pharmacia Co., Uppsala, Sweden. Other reagents were purchased from Wako Pure Chemical Co., Osaka, and Katayama Chemical Co., Osaka.

Measurement of PPO Activity. PPO activity was measured by a manometric method using a TAIYOO$_2$ up tester (Taitec Co., Tokyo). The reaction mixture consisted of 0.5 mL of 0.1 M aqueous solutions of various polyphenolics, 3 mL of 0.1 M potassium phosphate/0.1 M sodium phosphate (phosphate) buffer, pH 7.0, and 0.5 mL of the enzyme solution. After 10 min of incubation at 30 °C, oxygen uptake was determined.

On chromatography, PPO (PhO) activity was measured by a spectrophotometric method (Fujita et al., 1993) summarized as follows. The reaction mixture consisted of 0.5 mL of 20 mM aqueous solution of phloroglucinol; 1.3 mL of 0.1 M phosphate buffer, pH 7.0, and 0.2 mL of each fraction obtained by chromatography. After incubation for 10 min at 30 °C, 0.5 mL of the reaction mixture was taken out and added to 4.5 mL of distilled water, and the absorbance of the mixture was measured at 272 nm against a substrate blank. One unit of the enzyme activity was expressed as an increase in absorbance at 272 nm (ΔA_{272}) of 0.1 per minute in a 1 cm light path.

Measurement of POD Activity. POD activity was determined by a colorimetric method. The reaction mixture contained 0.5 mL of a 0.1 M aqueous solution of guaiacol, 4.1 mL of 0.1 M phosphate buffer, pH 6.0, 0.2 mL of 0.1% hydrogen peroxide, and 0.2 mL of the enzyme solution. After incubation for 2 min at 30 °C, the increase in absorbance at 470 nm (ΔA_{470}) of the mixture was measured. One unit of the enzyme activity was defined as 0.1 ΔA_{470}/min (1 cm light path).

Purification of the Enzyme. All steps were carried out at 5 °C. The whole cabbage (40 kg) were homogenized with 0.1 M phosphate buffer, pH 7.0. After filtration of the homogenate through cotton cloth, the filtrate was centrifuged at 7000*g* for 20 min and ammonium sulfate was added to the supernate. The protein fraction precipitating between 40 and 70% saturation with ammonium sulfate was collected by centrifugation, dissolved in a small volume of 0.01 M phosphate buffer, pH 7, and dialyzed overnight against the same buffer. The dialyzed solution was added to a DEAE-cellulose column (4.5 × 15 cm) equilibrated with the 0.01 M phosphate buffer, pH 7, and eluted with the same buffer. PPO fractions, which were not adsorbed on the column, were pooled, applied

* Author to whom correspondence should be addressed (fax 0952-22-6274).
† Saga University.
‡ Nishikyushu University.

0021-8561/95/1443-1138$09.00/0

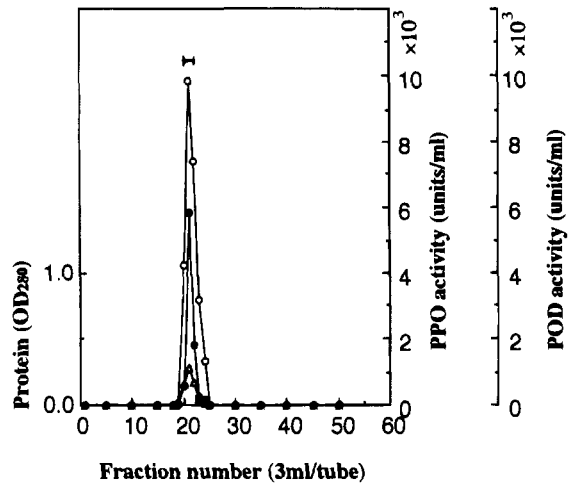

Figure 1. Elution pattern of the enzyme on Toyopearl HW 55F: (△) protein; (○) PPO activity; (●) POD activity; (|−|) fractions pooled.

to a CM-Sephadex C 50 column (2.1 × 60 cm) equilibrated with 0.01 M phosphate buffer, pH 7, and eluted with a linear gradient of sodium chloride (0−0.5 M NaCl in 0.01 M phosphate buffer, pH 7). The enzyme fractions were brought to contain 2 M ammonium sulfate and added to the butyl-Toyopearl 650M column (1.6 × 10 cm) equilibrated with 0.01 M phosphate buffer, pH 7, containing 2 M ammonium sulfate. The column was eluted with a linear gradient of ammonium sulfate (2−10 M ammonium sulfate in 0.01 M phosphate buffer, pH 7). The enzyme active fractions were pooled and dialyzed against 0.01 M phosphate buffer, pH 7. The dialyzed solution was concentrated with a membrane filter (Amicon Co., PM 10) and added to a Toyopearl HW 55F column (1.6 × 80 cm) equilibrated with 0.02 M phosphate buffer, pH 7, containing 0.2 M NaCl and eluted with the same buffer. The enzyme fractions were collected and concentrated by ultrafiltration, and the concentrated fraction was applied again to the Toyopearl HW 55F column. The PhO active fractions were pooled as purified PPO.

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 4.0.

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

Gel filtration was done following the method of Andrews (1965), using cytochrome *c* (MW 12 500), chymotrypsinogen A (25 000), egg albumin (45 000), bovine serum albumin (65 000), and γ-globulin (125 000) 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.

Amino Acid Analysis. The amino acid composition of the enzyme was analyzed according to the method of Spackman et al. (1958) with a JLC 300 amino acid analyzer (Nippon Denshi Co., Tokyo). The sample was dialyzed against distilled water and hydrolyzed at 110 °C in constant boiling HCl for 24, 48, and 72 h in sealed tubes.

Determination of Protein and Sugar Contents. Protein content was determined according to 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. Sugar content was determined according to the phenol−sulfuric acid method (Dubois et al., 1956) and expressed as glucose.

Determination of Metals. Metals in the purified enzyme solution were determined with a Perkin-Elmer 4100 ZL atomic absorption spectrophotometer.

RESULTS AND DISCUSSION

Purification of the Enzyme. Figure 1 shows the typical elution pattern of the enzyme activity on the final gel filtration. The enzyme activity showed one peak, and the peak fractions from several columns were pooled as the purified PPO.

A stepwise purification of the enzyme is given in Table 1. The increase of total enzyme activity after DEAE-cellulose chromatography might be caused by the removal of the enzyme inhibitor. Similar increments were found in the purifications of Satsuma mandarin (Fujita and Tono, 1979, 1980a) and turnip (Fujita and Tono, 1980b) enzymes, and the inhibitor was defined as a polyphenolic substance (Fujita and Tono, 1982). The purified cabbage enzyme was also inhibited by *o*- and *m*-polyphenols such as chlorogenic acid and hydroquinone (see Table 4). Finally, purification was about 410-fold with a recovery rate of 92%.

Some Properties of the Purified Enzyme. The purified enzyme was judged to be homogeneous by a single band produced on PAGE (see Figure 4) and SDS−PAGE (Figure 2). The band was stained by fuchsine (basic) as well as by Coomassie brilliant blue, and the enzyme solution contained 10.3% sugar as glucose. These results indicate that the cabbage PPO is a sugar protein. As shown in Figure 3, the molecular weight was estimated to be about 39 000 by gel filtration and about 40 000 by SDS−PAGE. These results indicate that the purified enzyme is a monomer. The molecular weight of the enzyme was larger than those of Satsuma Mandarin (Fujita and Tono, 1979) and turnip enzymes (Fujita and Tono, 1980b), both of which were estimated at about 27 000.

The amino acid composition of the purified enzyme is shown in Table 2. The values for amino acid residues were calculated from the molecular weight (40 000), deduced by the SDS−PAGE. Aspartic acid including asparagine was abundant in the enzyme molecule.

The substrate specificity of the purified cabbage PPO is different from most reported plant PPOs which markedly oxidize *o*-diphenols such as pyrocatechol, 4-methylcatechol, and chlorogenic acid (Rivas and Whitaker, 1973; Kahn, 1976, 1977; Wissemann and Lee, 1981; Augustin et al., 1985; Park and Luh, 1985; Fujita and Tono, 1988; Fujita et al., 1991; Murata et al., 1992). The purified enzyme strongly oxidized 1,3,5-trihydroxybenzenes such as phloroglucinol and phloroglucinolcarboxylic acid. However, the enzyme had no activity toward *o*-diphenols such as chlorogenic acid, pyrocatechol, DL-Dopa, and (−)-epicatechin or toward 1,2,3-trihydroxybenzenes such as pyrogallol and gallic acid. Similar substrate specificities were found in the purified enzymes of Satsuma mandarin (Fujita and Tono, 1979, 1980a) and turnip (Fujita and Tono, 1980b). Edible burdock PPO strongly oxidized 1,3,5-trihydroxybenzene; however, it also oxidized 1,2,3-trihydroxybenzenes (Murao et al., 1993). Considering the substrate specificity, the cabbage PPO seems to be a new type of PPO, "phloroglucinol oxidase (PhO)". The Michaelis constant (K_m) of the purified enzyme for phloroglucinol oxidation was 6.4 mM. The value was larger than that of Satsuma mandarin and turnip enzymes (0.67 mM).

The purified enzyme solution assumed a red color, and absorption maxima were detected at 280, 402, 490, and 630 nm. The same color and absorption maxima were found in Satsuma mandarin (Fujita and Tono, 1980a) and turnip PPO (Fujita and Tono, 1980b). Atomic absorption analysis indicated that the purified enzyme contained 1.58 μg of iron/mg of protein but was free from copper. This result indicates that the peaks

Table 1. Purification of the Cabbage Enzyme

	phloroglucinol oxidase					peroxidase			
	protein (mg)	total activity (kilounits)	specific activity (units/mg of protein)	purification (fold)	yield (%)	total activity (units)	specific activity (units/mg of protein)	purification (fold)	yield (%)
crude enzyme	13566	501	37	1.0	100	3957	292	1.0	100
DEAE-cellulose	5159	981	189	5.1	196	3912	754	2.6	99
CM-Sephadex C-50	230	893	3850	104.3	176	995	4336	14.9	25
butyl-Toyopearl 650M	57.6	793	12313	333.7	158	648	11250	38.6	16
Toyopearl HW55F (1st)	34.7	491	14123	382.7	98	412	10625	36.4	10
Toyopearl HW55F (2nd)	31.3	462	14817	401.5	92	333	11895	40.8	8

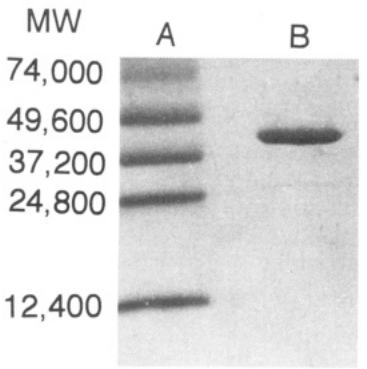

Figure 2. SDS–PAGE of the purified enzyme: (A) marker protein; (B) purified enzyme; (MW) molecular weight.

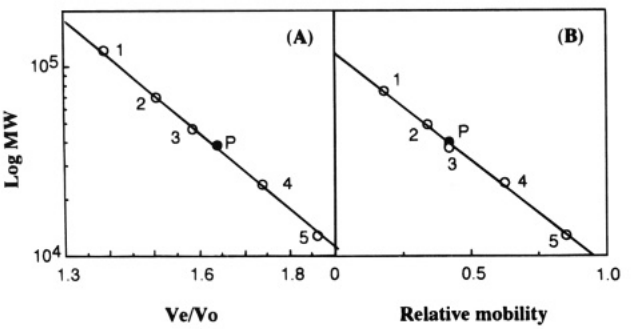

Figure 3. Molecular weight estimation of the enzyme by gel filtration on Toyopearl HW 55F (A) and SDS–PAGE (B). (A) V_0, void volume of the column; V_e, elution volume of the substance; MW, molecular weight in amu. 1, γ-Globulin; 2, bovine serum albumin; 3, egg albumin; 4, chymotrypsinogen A, 5; cytochrome *c*; P, purified enzyme. (B) MW, molecular weight in amu. Cytochrome *c*: 1, hexamer (74 000); 2, tetramer (49 600); 3, trimer (37 200); 4, dimer (24 800); 5, monomer (12 400); P, purified enzyme.

Table 2. Amino Acid Composition of the Enzyme

amino acid	residues/mol[a]	amino acid	residues/mol[a]
Asp[b]	48	Met[b]	4
Thr[c]	31	Ile[d]	12
Ser[c]	25	Leu[b]	36
Glu[b]	24	Tyr[b]	6
Pro[b]	19	Phe[b]	21
Gly[b]	22	His[b]	4
Ala[b]	28	Lys[b]	7
Cys[b]	3	Trp	ND[e]
Val[b]	20	Arg[b]	25

[a] Assuming the molecular weight to be 40 000. [b] Average value. [c] Extrapolated to zero hydrolysis time. [d] Maximum value. [e] Not determined.

(402, 490, and 630 nm) are caused by iron, as well as Satsuma mandarin PhO (Fujita and Tono, 1980a). The purified Satsuma mandarin and turnip enzymes had strong PhO and POD activities. Figure 4 shows that the purified enzyme gave a single band of protein on disc gel and that the PhO and POD activities were detected at the same position as that shown by protein

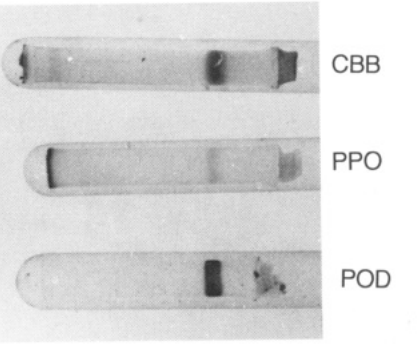

Figure 4. Polyacrylamide gel disc electrophoresis and activity staining of the purified enzyme. (CBB) Coomassie brillant blue; (PPO) phloroglucinol; (POD) guaiacol and H_2O_2.

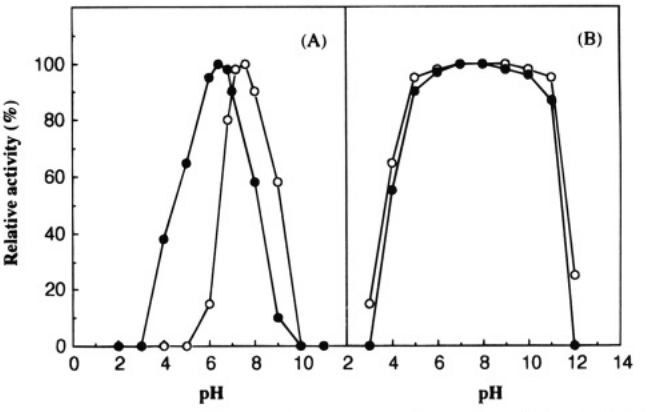

Figure 5. Effect of pH on the activity (A) and stability (B) of the enzyme: (○) PPO activity; (●) POD activity.

staining. The enzyme also gave a single band of protein on SDS–PAGE (Figure 2). Therefore, the purified cabbage enzyme also had dual activities of PhO and POD as well as Satsuma mandarin and turnip enzymes. Finally, purification was 41-fold with a recovery rate of 8% for POD (Table 1).

The effect of pH on PPO and POD activities is shown in Figure 5. The pH optima of the enzyme were 7.6 for phloroglucinol oxidation (PhO) and 6.4 for guaiacol (POD). Although the optimum pH of the purified enzyme for PPO almost agreed with that of Satsuma mandarin (Fujita and Tono, 1979, 1980a) and turnip enzymes (Fujita and Tono, 1980b), the optimum pH of the POD activity of the cabbage enzyme was slightly different from that of the latter two enzymes (pH 5.0). More than 90% of the original activities of both PPO and POD remained between pH 5 and 11 after 20 h of incubation at various pH values from 3 to 12.

Figure 6 shows the effect of temperature on both enzyme activities. The optimum temperatures of PPO and POD were 40 and 45 °C, respectively. Relatively high thermal stability was also found for the PPO in kiwi fruit (Park and Luh, 1985), mango (Park et al.,

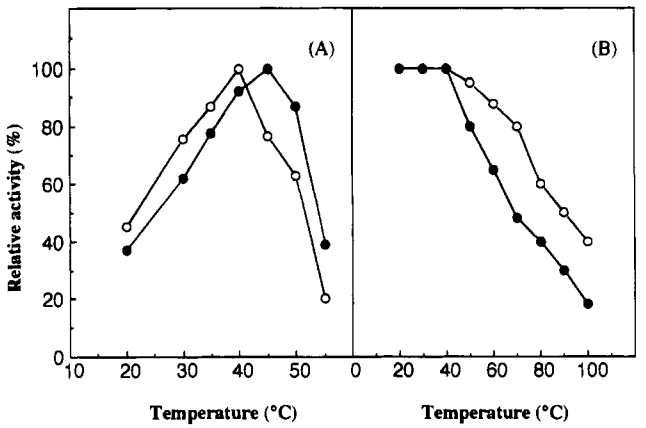

Figure 6. Effect of temperature on the activity (A) and stability (B) of the enzyme: (○) PPO activity; (●) POD activity.

Table 3. Effect of Various Compounds on PPO and POD Activities

	relative activity			
	PPO activity		POD activity	
compound	10 mM[a]	10 mM[a]	1 mM[a]	10 mM[a]
none	100	100	100	100
sodium diethyldithiocarbamate	0	0	0	0
potassium cyanide	73	36	0	0
EDTA	72	10	84	80
NaF	100	93	90	71
NaCl	100	87	92	84
$MnCl_2$	275	278	0	0
$CuSO_4$	0	0	65	60
$BaCl_2$	105	81	95	85
$ZnSO_4$	103	75	105	90
L-ascorbic acid	0	0	0	0
chlorogenic acid	8[b]	0[c]	46[b]	27[c]
resorcinol	100[b]	100[c]	93[b]	86[c]
hydroquinone	8[b]	2[c]	8[b]	1[c]

[a] Final concentration of compound. [b] Final concentration, 0.05 mM. [c] Final concentration, 0.5 mM.

1980), Satsuma mandarin (Fujita and Tono, 1979), and head lettuce (Fujita et al., 1991). In comparison with those PPO, the purified cabbage enzyme was highly heat stable: about 40% of the PPO activity remained after heat treatment at 100 °C for 10 min. POD was also highly thermally stable: about 50% of the POD activity remained after the heat treatment at 70 °C for 10 min. Similar thermal stabilities were found in the purified enzymes from Satsuma mandarin (Fujita and Tono, 1979) and turnip (Fujita and Tono, 1980b).

The effects of various compounds on PPO and POD activities are listed in Table 3. Both activities were markedly inhibited by metal enzyme inhibitor such as sodium diethyldithiocarbamate and potassium cyanide and by L-ascorbic acid. PPO was also inhibited by EDTA and $CuSO_4$, but POD was a little inhibited. $MnCl_2$ markedly activated PPO but strongly inhibited POD. NaCl, NaF, $BaCl_2$, and $ZnSO_4$ were poor inhibitors of both enzymes. Similar effects of these compounds were found on the purified PPO and POD from Satsuma mandarin (Fujita and Tono, 1979) and turnip (Fujita and Tono, 1980a,b).

Considering these different effects of pH, temperature, and various compounds on PPO and POD activities of the purified cabbage enzyme, it appears that our enzyme may have separate sites for these activities.

LITERATURE CITED

Andrew, P. The gel-filtration behavior of proteins related to their molecular weight over wide range. *Biochem. J.* **1965**, *96*, 595–605.

Augustin, M. A.; Ghazali, H. M.; Hashim. H. Polyphenoloxidase from guava (*Psidium guajava* L.). *J. Sci. Food Agric.* **1985**. *36*, 1259–1265.

Chilaka, F. C.; Amosike, E. O.; Egubuna, P. C. Purification and properties of polyphenol oxidase from oil bean (*Pentaclethra macrophylla* Benth) seeds. *J. Sci. Food Agric.* **1993**, *61*, 125–127.

DaDamio, P. A.; Thompson, D. B. Mushroom (*Agaricus bisporus*), its polyphenoloxidase, and polyphenolics affected *in vitro* iron availability. *J. Food Sci.* **1992**, *57*, 458–461.

Davis, B. J. Disc gel electrophoresis. 2. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **1964**, *121*, 404–427.

Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rabers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356.

Fujita, S.; Tono, T. Purification of pyrogalloloxidase and phloroglucinoloxidase from Satsuma mandarin fruits and their properties. *Nippon Nogeikagaku Kaishi* **1979**, *53*, 233–240.

Fujita, S.; Tono, T. Peroxidase activity of phloroglucinoloxidase from Satsuma mandarin fruits and effect of metal ions on the enzyme activity. *Nippon Nogeikagaku Kaishi* **1980a**, *54*, 201–208.

Fujita, S.; Tono, T. Purification of phloroglucinoloxidase from turnip and its properties. *Nippon Nogeikagaku Kaishi* **1980b**, *54*, 429–435.

Fujita, S.; Tono, T. Inhibitory effect of polyphenols on phloroglucinoloxidase activity from Satsuma mandarin. *Bull. Fac. Agric. Saga Univ.* **1982**, *53*, 17–26.

Fujita, S.; Tono, T. Purification and some properties of polyphenol oxidase in eggplant (*Solanum melongena*). *J. Sci. Food Agric.* **1988**, *46*, 115–123.

Fujita, S.; Tono, T.; Kawahara, H. Purification and properties of polyphenol oxidase in head lettuce (*Lactuca sativa*). *J. Sci. Food Agric.* **1991**, *55*, 643–651.

Fujita, S.; Kawahara, H.; Nazamid, S.; Tono, T. *Spectrophotometric* determination of phloroglucinoloxidase activity based on difference spectra. *Bull. Fac. Agric. Saga Univ.* **1993**, *74*, 81–88.

Hartree, E. F. Determination of protein: a modification of Lowry method that gives a linear photometric response. *Anal. Biochem.* **1972**, *48*, 422–427.

Kahn, V. Polyphenol oxidase in avocado. *Phytochemistry* **1976**, *15*, 267–272.

Kahn, V. Some biochemical properties of polyphenoloxidase from two avocado varieties differing in their browning rate. *J. Food Sci.* **1977**, *42*, 38–43.

Luh, B. S.; Phithakpol, B. Characteristics of polyphenoloxidase related to browning in cling peach. *J. Food Sci.* **1972**, *27*, 264–268.

Murao, S.; Oyama, H.; Nomura, Y.; Tono, T.; Shin, T. Purification and characterization of *Arctium lappa* L. (Edible burdock) polyphenol oxidase. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 177–180.

Murata, M.; Kurokami, C.; Homma, S. Purification and properties of chlorogenic acid oxidase from apple (*Malus pumula*). *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1705–1710.

Nawa, R.; Hosoda, H.; Shiina, T.; Ito, H.; Kurogi, M. Quality evaluation and preservation of shredded vegetables. 2. Charges in total phenol content and polyphenol oxidase activity in different cultivars of cabbage during storage after shredding. *Rep. Natl. Food Res. Inst.* **1987**, *50*, 65–69.

Park, E. Y.; Luh, B. S. Polyphenol oxidase of kiwi fruit. *J. Food Sci.* **1985**, *50*, 679–684.

Park, Y. K.; Sato, H. H.; Almeid, T. D.; Moretti, K. H. Polyphenol oxidase of mango. *J. Food Sci.* **1980**, *45*, 1619–1621.

Rivas, N. J.; Whitaker, J. R. Purification and some properties of two polyphenol oxidase from bartlett pears. *Plant Physiol.* **1973**, *52*, 501–507.

Spackman, D. H.; Stein, W. H.; Moore, S. Automatic recording apparatus for use in chromatography of amino acid. *Anal. Chem.* **1958**, *30*, 1190–1206.

Tono, T.; Fujita, S.; Kawasaki, H.; Li, Z. Purification and high L-epicatechin oxidase activity in polyphenol oxidase of Japanese pear. *Nippon Nogeikagaku Kaishi* **1986**, *60*, 705–712.

Weber, K.; Osborn, M. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **1969**, *244*, 4406–4412.

Wissemann, K. W.; Lee, C. Y. Characterization of polyphenoloxidase from Rovat 51 and Niagara grapes, *J. Food Sci.* **1981**, *46*, 506–508.

Received for review November 4, 1994. Accepted March 20, 1995.[⊗]

JF940623U

[⊗] Abstract published in *Advance ACS Abstracts,* April 15, 1995.