Isolation and Characterization of Two Phloroglucinol Oxidases from Cabbage (*Brassica oleracea* L.)

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Two isozymes (F-IB and F-II) of phloroglucinol oxidase (PhO) in cabbage (*Brassica oleracea* L.) were purified from cabbage. The purified enzymes were found to be in a homogeneous state by polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE. The molecular weights of F-IB and F-II were estimated to be ~43 000 and 32 000, respectively, by SDS-PAGE. Both purified enzymes only oxidized 1,3,5-trihydroxybenzenes, such as phloroglucinol and phloroglucinolcarboxylic acid. Both enzymes also had strong peroxidase (POD) activity. The pH optima of PhO and POD of F-IB were 8.0 and 6.7, respectively, and those of F-II were 7.4 and 6.7, respectively. Activities of both F-IB and F-II were stable in the pH range 6−11 at 5 °C for 20 h, and were markedly inhibited by sodium diethyldithiocarbamate and potassium cyanide. $MnCl_2$ markedly activated the PhO activity of F-IB and F-II, but strongly inhibited their POD activity.

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

INTRODUCTION

In fresh fruit and vegetables, undesirable browning of damaged tissue occurs by enzymatic oxidation of polyphenols. Such oxidation is mainly caused by polyphenol oxidase (EC 1.10.3.1, PPO). To prevent the browning that results in decreased marketability of products, these enzymes have been characterized in many fruits and vegetables (Luh and Phithakpool, 1972; Kahn, 1976, and 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). Most of the PPOs strongly oxidized *o*-diphenols, such as pyrocatechol, 4-methylcatechol, chlorogenic acid, and DL-dopa. In contrast, we purified and characterized a novel type of PPO from cabbage (which was called F-IA) that only oxidized 1,3,5-trihydroxybenzenes, such as phloroglucinol and phloroglucinolcarboxylic acid, and found that the purified enzyme also had strong peroxidase (POD) activity (Fujita et al., 1995). Similar dual activities of phloroglucinol oxidase (PhO) and POD were detected in the purified enzymes of Satsuma mandarin and turnip (Fujita and Tono, 1980a,b). In addition, we found another two isozymes of the phloroglucinol oxidizing enzymes, which were called F-IB and F-II, in cabbage. It is interesting that the latter two isozymes (F-IB and F-II) have dual activities of PhO and POD as well as Satsuma mandarin, turnip, and cabbage (F-IA) enzymes (Fujita and Tono, 1980a,b; Fujita et al., 1995). In the present study, the latter two isozymes of PPO were purified to a homogeneous state from cabbage, and some properties of the purified enzymes were investigated.

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MATERIALS AND METHODS

Materials. Fresh cabbage (*Brassica oleracea* L.) was purchased from a local market in Saga City. DEAE-cellulose (DE23) was obtained from Whatman Company, Maidstone Kent, U.K. Butyl-Toyopearl 650M and Toyopearl HW55F were obtained from Toso Company, Tokyo, Japan. Cellulofine GCL-2000 was purchased from Chisso Company, Tokyo, Japan, and CM-Sephadex C-50 was obtained from Pharmacia Company, Uppsala, Sweden. Other reagents were purchased from Wako Pure Chemical Company, Osaka, Japan, and Katayama Chemical Company, Osaka, Japan.

Measurement of PPO Activity. PPO activity was measured by a manometric method using a TAIYO O_2 up tester (Taitec Company, Tokyo, Japan). The reaction mixture consisted of 0.5 mL of 0.1 M aqueous solutions of various polyphenolics listed in Table 2 (final concentration, 12.5 mM), 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.

PPO (PhO) activity was measured by a spectrophotometric method (Fujita et al., 1993). 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 min 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} per min (1 cm light path).

Purification of the Enzyme. All steps were carried out at 5 °C. The whole cabbage (40 kg) was homogenized with

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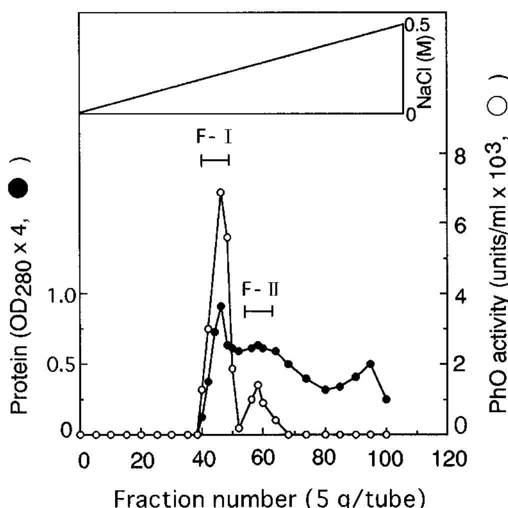

Figure 1. Elution pattern of the enzyme on CM-Sephadex C-50; (-) fraction pooled.

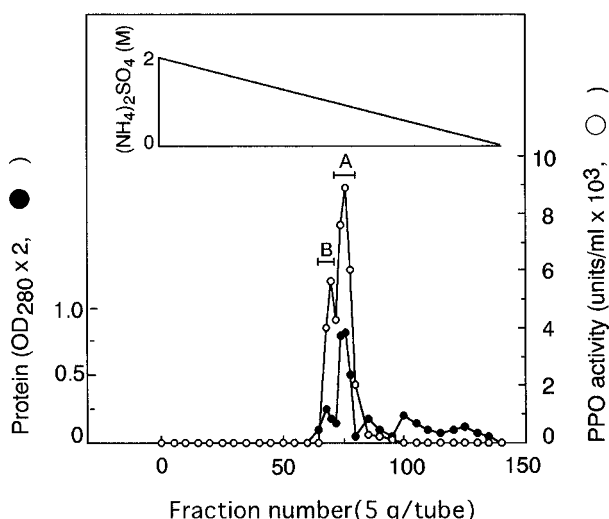

Figure 2. Elution pattern of F-I on butyl-Toyopearl 650M; (-) fraction pooled.

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) that was equilibrated with the 0.01 M phosphate buffer (pH 7), and eluted with the same buffer. PhO fractions, which were not adsorbed on the column, were pooled, applied to a CM-Sephadex C-50 column (2.1 × 60 cm) that was equilibrated with 0.01 M phosphate buffer (pH 7), and eluted with a linear gradient of sodium chloride (0 to 0.5 M NaCl in 0.01 M phosphate buffer, pH 7). As shown in Figure 1, two PhO fractions (F-I and F-II) were obtained, and the each enzyme fraction was brought to contain 2 M ammonium sulfate individually.

F-I was 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, and the column was eluted with a linear gradient of ammonium sulfate (2 to 0 M ammonium sulfate in 0.01 M phosphate buffer, pH 7). Two active enzyme fractions (F-IA and F-IB) were obtained, as shown in Figure 2. These fractions were pooled and dialyzed against 0.01 M phosphate buffer (pH 7). Further purification and characterization of F-IA were done as reported in the previous paper (Fujita et al., 1995). The dialyzed solution of F-IB was concentrated with a membrane filter (Amicon Company, PM 10), added to a Toyopearl HW55F column (1.6 × 80 cm) that was 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 HW55F column. The active enzyme fractions were pooled as purified PhO F-IB.

On the other hand, F-II was added to the same butyl-Toyopearl column and eluted with the same conditions as used for F-IB. The PhO active fractions were pooled and dialyzed against 0.02 M phosphate buffer (pH 7). The dialyzed solution was concentrated with a membrane filter, added to a Cellulofine GCL-2000 column (1.6 × 42 cm) that was equilibrated with 0.02 M phosphate buffer (pH 7) containing 0.2 M NaCl, and eluted with the same buffer. The active enzyme fractions were pooled as purified PhO F-II.

Polyacrylamide Gel Electrophoresis (PAGE). Electrophoresis of the purified enzyme was carried out according to the method of Davis (1964), with 7.5% polyacrylamide gel at pH 4.0.

Molecular Weight Determination. Molecular weight of the purified enzyme was determined by gel filtration and sodium dodecyl sulfate (SDS)-PAGE. Gel filtration was done following the method of Andrews (1965), with cytochrome c (MW, 12 500), chymotrypsinogen A (25 000), egg albumin (45 000), bovine serum albumin (65 000), and γ-globulin (12 500) as marker proteins. SDS-PAGE was carried out as described by Weber and Osborn (1969), with an SDS marker protein kit (Oriental Yeast Company, Tokyo, Japan) as standard.

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

RESULTS AND DISCUSSION

Purification of the Enzyme. A typical stepwise purification of PhO F-IB and F-II 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 major cabbage enzyme (F-IA) was also inhibited by *o*- and *m*-polyphenols, such as chlorogenic acid and hydroquinone, as reported in a previous paper (Fujita et al., 1995). The final purifications of F-IB and F-II for PhO were ~430-fold, with a recovery rate of 29%, and ~106-fold, with a recovery rate of 4%, respectively. Both of the purified enzymes, F-IB and F-II, as well as F-IA had strong POD activity (Fujita et al., 1995). Finally, purifications of FI-B and F-II for POD were 32-fold, with a recovery rate of 2%, and 272-fold, with a recovery rate of 10%, respectively (Table 1). The specific activity of F-II was highest for POD, but smallest for PhO of all cabbage PPOs.

Some Properties of the Purified Enzyme. Both purified enzymes were judged to be homogeneous by a single band produced on PAGE and SDS-PAGE (Figure 3). The bands of both enzymes were also stained by fuchsine (basic) as well as by Coomassie brilliant blue. These results indicate that F-IB and F-II, like F-IA, were sugar proteins (Fujita et al., 1995).

Enzymatic and physicochemical properties of F-IB and F-II are summarized in Tables 2 and 3. The data of F-IA (Fujita et al., 1995) were also added in these tables. The molecular weights of F-IB and F-II were estimated to be ~45 000 and 33 000 by gel filtration, and ~43 000 and 32 000 by SDS-PAGE, respectively.

Table 1. Purification of the Cabbage Enzyme (F-IB and F-II)

	phloroglucinol oxidase					peroxidase			
enzyme	protein (mg)	total activity (kilo unit)	specific activity (unit/mg protein)	purification (fold)	yield (%)	total activity (kilo unit)	specific activity (unit/mg 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
F-IB									
CM-Sephadex C-50	230	893	3850	104.3	176	995	4336	14.9	25
butyl Toyopearl 650M	13.4	184	13582	368.1	37	129	9513	32.6	3
Toyopearl HW55F (1st)	9.3	144	14817	419.8	29	97	10411	35.7	2
Toyopearl HW55F (2nd)	9.0	143	15489	428.7	29	84	9318	31.9	2
F-II									
CM-Sephadex C-50	178	54	301	8.2	11	1050	5893	20.2	26
butyl Toyopearl 650M	7.1	22	3133	84.9	4	942	50667	173.7	24
Cellulofine GCL-2000	4.9	19	3900	105.7	4	388	79200	271.5	10

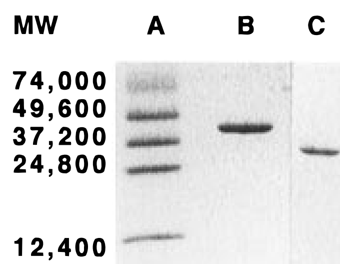

Figure 3. SDS-PAGE of the purified enzymes: (A) marker protein; (B) F-IB; (C) F-II; (MW) molecular weight.

Table 2. Some Properties of the Purified Enzymes

property	F-IB	F-II	F-IA[c]
molecular weight			
gel filtration	45 000	33 000	39 000
SDS-PAGE	43 000	32 000	40 000
substrate specificity (%)[a]			
phloroglucinol	100	100	100
phloroglucinolcarboxylic acid	58	49	52
chlorogenic acid	0	0	0
pyrocatechol	0	0	0
resorcinol	0	0	0
hydroquinone	0	0	0
Michaelis constant (mM)[b]	8.5	1.3	6.4

[a] Relative activity. [b] For phloroglucinol oxidation. [c] The data of F-IA are quoted from our previous paper (Fujita et al., 1995).

These results indicate that all the purified enzymes are monomers and the molecular weight of the purified enzymes were in the order F-IB > F-IA > F-II. All of the cabbage PhOs were larger than those of Satsuma mandarin and turnip enzymes, both of which were estimated at ~27 000 (Fujita and Tono, 1980a,b).

The substrate specificity of all the purified cabbage PPOs is different from most reported plant PPOs that markedly oxidize *o*-diphenols, such as pyrocatechol, 4-methylcatechol, and chlorogenic acid (Rivas and Whitaker, 1973; Kahn, 1976 and 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 enzymes (F-IB and F-II) strongly oxidized 1,3,5-trihydroxybenzenes, such as phloroglucinol and phloroglucinolcarboxylic acid. However, these enzymes had no activity towards *o*-diphenols, such as chlorogenic acid, pyrocatechol, DL-dopa and (−)-epicatechin, and towards 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 and 1980a) and turnip (Fujita and Tono, 1980b). Edible burdock PPO strongly oxidized 1,3,5-trihydroxybenzenes; however, it also oxidized 1,2,3-trihydroxybenzenes (Murao et al., 1993). Such substrate specificity indicates that all of the purified cabbage PPOs seem to be of a novel type PPO; that is, phloroglucinol oxidase (PhO). The Michaelis constants (K_M) of the F-IB and F-II for phloroglucinol oxidation were 8.5 and 1.3 mM, respectively. The values were larger than those of Satsuma mandarin and turnip enzymes (0.67 mM). Phloroglucinol had been isolated as an aglycone from fruits of orange (Horowitz and Gentili, 1961), lemon (Vondercook and Stephanson, 1966), and grape fruit (Horowitz and Gentili, 1961,

Table 3. Properties of the Purified Enzymes

	phloroglucinol oxidase activity						peroxidase activity					
property	F-IB		F-II		F-IA[e]		F-IB		F-II		F-IA[e]	
optimum pH	8.0		7.4		7.4		6.7		6.7		6.4	
pH stability[a]	6-11		6-11		5-11		6-11		6-10		5-11	
heat stability (°C)[b]	62		65		83		57		53		69	
effect of compounds	1 mM[d]	10 mM[d]	1 mM[d]	10 mM[d]	1 mM[d]	10 mM[d]	1 mM[d]	10 mM[d]	1 mM[d]	10 mM[d]	1 mM[d]	10 mM[d]
sodium diethyldithiocarbamate	0	0	0	0	0	0	0	0	0	0	0	0
potassium cyanide	80	41	40	28	73	36	0	0	0	0	0	0
EDTA-2Na	0	0	70	10	72	10	88	77	87	75	92	84
L-ascorbic acid	0	0	0	0	0	0	0	0	0	0	0	0
$MnCl_2$	425	425	400	481	275	278	46	10	63	10	45	10
$CuSO_4$	0	0	82	0	0	0	63	50	70	71	65	65
$Bacl_2$	97	111	113	112	108	81	93	77	90	81	95	86
$ZnSO_4$	89	75	115	65	013	75	105	85	100	76	110	90

[a] Above 90% remaining activity. [b] 50% remaining activity after incubation at pH 7.0 for 10 min. [c] The values given are percent of contro (contained no effecters was assigned an activity of 100%). [d] Final concentration of the compound. [e] The data of F-I A are quoted from our previous paper (Fujita et al., 1995).

Maier and Metzler, 1967). Tono et al. (1973) indicated that phloroglucinol was a more suitable substrate for enzymatic browning than any other polyphenolics in citrus fruits. Although phloroglucinol has not been detected in cabbage, it seems that our three PhOs may play an important role in the enzymatic browning in cabbage because of their substrate specificity.

The purified enzyme solutions of F-IB and F-II assumed a red color, with absorption maxima at 280, 402, 490, and 630 nm. The same color and absorption maxima were also found in F-IA, in which iron was detected but was free from copper, as shown by atomic absorption analysis (Fujita et al., 1995). The same results were obtained in Satsuma mandarin (Fujita and Tono, 1980a) and turnip PPO (Fujita and Tono, 1980b) in which only iron was detected. From these results, the peaks (402, 490, and 630 nm) of F-IB and F-II appear to be caused by iron, as well as cabbage PPO F-IA and Satsuma mandarin PhO (Fujita and Tono, 1980a). The purified Satsuma mandarin, turnip enzymes, and cabbage PPO F-IA had strong PhO and POD activities (Fujita and Tono, 1980a,b; Fujita et al., 1995). Both of the purified enzymes (F-IB and F-II) had strong POD activity as well as PhO activity. Because both enzymes were homogeneous by SDS-PAGE, the enzymes had dual activities of PhO and POD similar to Satsuma mandarin, turnip enzymes, and PPO F-IA.

The pH optima of the F-IB and F-II were 8.0 and 7.4, respectively, for phloroglucinol oxidation (PhO) and 6.7 and 6.7, respectively, for guaiacol (POD). Although the optimum pH of all purified cabbage enzymes for PhO were almost identical with the Satsuma mandarin (Fujita and Tono, 1979, 1980a) and turnip enzymes (Fujita and Tono, 1980b), the optimum pH of the POD activity of the cabbage enzymes were slightly different from that of the latter two enzymes (pH 5.0). More than 90% of the original activities of both PPO and POD of F-IB and F-II remained between pH 6 and pH 11 after 20 h of incubation at various pH values from 3 to 12.

Relatively high thermal stability was also found for the PPO 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). In comparison with those PPO, the purified cabbage enzymes were more heat stable; 50% of the PhO activities of F-IA, F-IB, and F-II was retained after heat treatment at 83, 62 and 65 °C for 10 min, respectively. POD activities of F-IA, F-IB, and F-II were also highly thermally stable; 50% of their POD activity remained after the heat treatment at 69, 57, 53 °C for 10 min, respectively. Similar thermal stabilities were found in the purified enzymes from Satsuma mandarin (Fujita and Tono, 1979) and turnip (Fujita and Tono, 1980b).

Both activities of all cabbage PhOs were markedly inhibited by metal enzyme inhibitors, such as sodium diethyldithiocarbamate and potassium cyanide, and by L-ascorbic acid. PhO was also inhibited by EDTA and $CuSO_4$, but POD was slightly inhibited. $MnCl_2$ markedly activated PhO but strongly inhibited POD. NaCl, NaF, $BaCl_2$, and $ZnSO_4$ were poor inhibitors of both activities of all enzymes. Similar effects of these compounds were found (Fujita and Tono, 1980a,b) on both activities of the purified enzymes from Satsuma mandarin and turnip.

In conclusion, three isozymes of PhO, which had different molecular weights, were isolated from cabbage. All of the purified enzymes had dual activities of PhO and POD; however, according to the specific activity of each enzyme, F-IA and F-IB appeared to behave as a PhO rather than a POD, whereas F-II behaves as a POD rather than a PhO. In addition, on the basis of different effects of pH, temperature, and various compounds on both activities of all enzymes, all of our enzymes may have separate active sites for PhO and POD activities.

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