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Purification and Characterization of Polyphenol Oxidase from Cauliflower (*Brassica oleracea* L.)

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ABSTRACT: Polyphenol oxidase (PPO) of cauliflower was purified to 282-fold with a recovery rate of 8.1%, using phloroglucinol as a substrate. The enzyme appeared as a single band on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The estimated molecular weight of the enzyme was 60 and 54 kDa by SDS−PAGE and gel filtration, respectively. The purified enzyme, called phloroglucinol oxidase (PhO), oxidized phloroglucinol (K_m = 3.3 mM) and phloroglucinolcarboxylic acid. The enzyme also had peroxidase (POD) activity. At the final step, the activity of purified cauliflower POD was 110-fold with a recovery rate of 3.2%. The PhO and POD showed the highest activity at pH 8.0 and 4.0 and were stable in the pH range of 3.0−11.0 and 5.0−8.0 at 5 °C for 20 h, respectively. The optimum temperature was 55 °C for PhO and 20 °C for POD. The most effective inhibitor for PhO was sodium diethyldithiocarbamate at 10 mM (IC_{50} = 0.64 and K_i = 0.15 mM), and the most effective inhibitor for POD was potassium cyanide at 1.0 mM (IC_{50} = 0.03 and K_i = 29 μM).

KEYWORDS: *Cauliflower, polyphenol oxidase, phloroglucinol oxidase, peroxidase, purification, characterization*

INTRODUCTION

Polyphenol oxidase (EC 1.10.3.1; *o*-diphenol:oxygen oxidoreductase, PPO) oxidizes phenolic compounds to produce undesirable browning of damaged tissues in many fruits and vegetables. Their quality and marketability decrease because of this type of browning during storage and processing. To prevent such browning, PPO has been widely investigated in many fruits and vegetables.[1−8] Most of the PPOs studied strongly oxidized *o*-diphenols, such as catechol, dopamine, and chlorogenic acid. The PPOs of apple,[1] garland chrysanthemum,[2] and edible burdock[3] strongly oxidized chlorogenic acid. In addition, banana PPO[4,5] strongly oxidized dopamine, and the PPOs of mango[6] and mamey[7] strongly oxidized catechol. A new type of PPO, which oxidizes only 1,3,5-trihydroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid, has been found in Satsuma mandarin,[9] turnip,[10] and cabbage.[11,12] The phloroglucinol oxidizing enzyme (PhO) also has strong peroxidase (EC 1.11.1.7; POD) activity.[9−12] The PPO and POD play important roles in the deterioration of color and flavor. As discussed by Rayan et al.,[13] the PPO is responsible for browning in many fruits and vegetables. On the other hand, Japanese radish PPO[14] and soybean PPO[15] oxidize not only 1,3,5-trihydroxybenzenes but also 1,2,3-trihydroxybenzenes, such as pyrogallol and gallic acid. These enzymes also have POD activity. We found that the crude enzyme extract of cauliflower (*Brassica oleracea* L.), which belongs to cruciferae vegetables, including cabbage and Japanese radish, had strong PhO and POD activity. The cauliflower POD has been purified and characterized, in which the result showed that the cauliflower is a good resource of POD.[16] The cauliflower enzymes decrease the quality of this vegetable during storage and processing in an appropriate condition. Determination of PPO and POD is important to identify its biochemical properties and function and, in turn, to understand how to prevent its deteriorative action during storage and processing. However, little is known about the characteristics of cauliflower enzyme PPO. In the present study, we purified the PPO of cauliflower using phloroglucinol as the substrate and characterized the PPO and POD activities of the purified enzyme.

MATERIALS AND METHODS

Chemicals and Materials. Fresh cauliflower (*B. oleracea* L.), which was purchased from a local market in Saga City, Japan, was used. DEAE-Toyopearl 650-M, CM-Sephadex C-50, butyl-Toyopearl 650-M, and Toyopearl HW 55-s were obtained from Tosoh Co., Tokyo, Japan. Other reagents were purchased from Wako Pure Chemical Company, Osaka, Japan.

Measurement of the Enzyme Activity. *PhO Activity.* The activity of PhO was measured by the spectrophtometric method based on a difference in spectra.[17] The reaction mixture consisted of 0.5 mL of 20 mM aqueous solution of phloroglucinol, 1.4 mL of 0.1 M potassium phosphate/0.1 M sodium hydrogen phosphate buffer (phosphate buffer at pH 7.0), and 0.1 mL of each fraction obtained by chromatography. After 10 min of incubation at 30 °C, 0.5 mL of the reaction mixture was taken out and added to 4.5 mL of distilled water. Immediately after being added to water, the enzyme activity was measured at 272 nm against an enzyme blank. A total of 1 unit of enzyme activity was defined as a change in absorbance of the mixture at 272 nm (ΔA_{272}) of 0.1 per minute and per milliliter of enzyme solution (1.0 cm light path).

PPO Activity. The activity of PPO was measured by the colorimetric method.[4] The reaction mixture consisted of 0.5 mL of 10 mM aqueous solution of various polyphenols, 4.0 mL of 0.1 M phosphate buffer (pH 7.0), and 0.5 mL of enzyme solution. After 5 min of incubation of

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the mixture at 30 °C, the increase in absorbance was measured at 420 nm. A total of 1 unit of enzyme activity was defined as a change in absorbance of the mixture at 420 nm (ΔA_{420}) of 0.1 per minute and per milliliter of enzyme solution (1.0 cm light path).

POD Activity. The activity of POD was determined by the colorimetric method.[11] The reaction mixture consisted of 0.5 mL of 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 2 min of incubation of the mixture at 30 °C, the increase in absorbance at 470 nm was measured. A total of 1 unit of enzyme activity was defined as a change in absorbance of the mixture at 470 nm (ΔA_{470}) of 0.1 per minute and per milliliter of enzyme solution (1.0 cm light path).

Assay of Enzyme Properties. *Optimum pH.* The activity of PhO and POD was measured at 30 °C in 0.2 M sodium phosphate/0.1 M citric acid buffer (McIlvaine buffer) in the pH range of 3.0−8.0 and also in 0.1 M boric acid−potassium chloride/0.1 M sodium carbonate (Atkins−Pantin buffer) in the pH range of 9.0−11.0. The enzyme activity was expressed as the percentage of maximum activity.

pH Stability. The enzyme of cauliflower was pre-incubated at 5 °C for 20 h in McIlvaine buffer in the pH range of 3.0−8.0 and in Atkins−Pantin buffer in the pH range of 9.0−11.0. The remaining PhO and POD activity was measured under the standard conditions (PhO, pH 7.0 and 30 °C; POD, pH 6.0 and 30 °C). The enzyme activity was defined as the percentage of the maximum activity level.

Optimum Temperature. The activity of PhO and POD was measured at pH 7.0 in the temperature range of 20−80 °C and at pH 6.0 in the temperature range of 10−60 °C, respectively. The enzyme activity was defined as the percentage of the maximum activity level.

Thermal Stability. Enzyme solution was preheated for 10 min at 20−80 °C. The remaining activity of PhO and POD was measured under the standard conditions (PhO, pH 7.0 and 30 °C; POD, pH 6.0 and 30 °C). The enzyme activity was defined as the percentage of the maximum activity level.

Effect of Various Compounds. The activity of PhO and POD was measured in the absence of 13 kinds of compounds, shown in Table 3, under the standard conditions (PhO, pH 7.0 and 30 °C; POD, pH 6.0 and 30 °C).

Determination of Protein. The protein content was determined according to the method by Lowry, which was modified by Hartree[18] using bovine serum albumin (BSA, fraction V, Katayama Chemical Company, Osaka, Japan) as a standard. In chromatography, protein was measured at an absorbance of 280 nm.

Molecular-Weight Determination. The molecular weight of the purified enzyme was determined by gel filtration and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The gel filtration was carried out following the method by Andrews,[19] using α-globulin (160 kDa), serum albumin (bovine, 65 kDa), ovalbumin (chicken egg, 44 kDa), and cytochrome *c* (12.4 kDa) as marker proteins. The SDS−PAGE was carried out as described by Weber and Osborn,[20] with myosin (209 kDa), β-galactosidase (124 kDa), serum albumin (bovine, 80 kDa), ovalbumin (chicken egg, 49.1 kDa), carbonic anhydrase (34.8 kDa), and soybean trypsin inhibitor (28.9 kDa) as marker proteins. The proteins were stained by 0.25% Coomassie Brilliant Blue (CBB).

Purification of Cauliflower Enzyme. The edible parts of cauliflower (8.0−9.0 kg) were homogenized with 0.1 M phosphate buffer (pH 7.0) at 5 °C. After filtration of the homogenate through a cotton cloth, the filtrate was centrifuged at 10300*g* for 20 min at 5 °C and the supernatant was brought to 80% ammonium sulfate saturation. After 24 h, the precipitated protein was collected by centrifugation (10300*g*), dissolved in a small volume of 0.01 M phosphate buffer (pH 7.0), and then dialyzed at 5 °C in the same buffer for 36 h, during which the buffer was changed 4 times.

For further purification of PPO, the dialyzed enzyme solution was added to a DEAE-Toyopearl 650-M column (4.5 × 10 cm), equilibrated with 0.01 M phosphate buffer (pH 7.0), and eluted with the same buffer. The active fractions of PPO were pooled and applied to a CM-Sephadex C-50 column (4.5 × 10 cm), equilibrated with 0.01 M phosphate buffer (pH 7.0), and eluted with a linear gradient of sodium chloride from 0 to 1.0 M in 0.01 M phosphate buffer (pH 7.0). The PPO active fraction was collected and brought to a 1.0 M ammonium sulfate concentration. Then, the collected fraction was applied to a butyl-Toyopearl 650-M column (1.6 × 15 cm), equilibrated with 0.01 M phosphate buffer (pH 7.0) containing 1.0 M ammonium sulfate, and eluted with a linear gradient of ammonium sulfate from 1 to 0 M in 0.01 M phosphate buffer (pH 7.0). The enzyme active fraction was pooled, dialyzed with 0.01 M phosphate buffer (pH 7.0), and concentrated with a membrane filter (Amicon YM-10, Millipore Japan Co., Tokyo, Japan).

The final purification was performed by a Toyopearl HW 55-s column (1.6 × 80 cm). After concentration, the solution was added to the column, equilibrated with 0.1 M phosphate buffer (pH 7.0), and eluted using the same buffer. The PPO active fraction was pooled as the purified enzyme of cauliflower and used for enzyme characterization.

Statistical Analysis. All experiments were conducted in triplicate, and all of the measurements were performed in triplicate. Standard deviation of the data was analyzed.

RESULTS AND DISCUSSION

Purification of the Enzyme. The enzyme of cauliflower was purified by ion-exchange chromatography, hydrophobic chromatography, and gel filtration using phloroglucinol as a substrate. Figure 1 shows a typical elution pattern of the enzyme on a final gel filtration. The enzyme activity showed a sharp single peak on the column. The active fraction of enzyme from several columns was pooled as the purified enzyme. The activity and yield of PPO in the course of purification are presented in Table 1. Finally, the PPO was purified up to 282 ± 16.5-fold purification with a recovery rate of 8.1 ± 0.5%, as compared to the crude extract. The obtained enzyme was used for further studies.

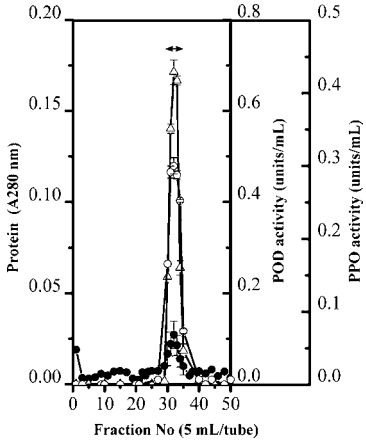

Figure 1. Elution pattern of the cauliflower enzyme on a Toyopearl HW 55-s column: (↔) fraction pooled, (○) PhO activity, (●) protein, and (△) POD activity.

Characterization of the Purified Enzyme. As shown in Figure 2, the purified enzyme of cauliflower produced a single band on SDS−PAGE. The molecular weight of the enzyme was estimated to be 60 ± 0.036 kDa by SDS−PAGE and 54 ± 0.025 kDa by gel filtration (Figure 3). This result indicates that the purified enzyme is a monomer protein. The molecular weight of the purified cauliflower PPO was different from the molecular weight of the PPOs of another plant, such as edible burdock (40 kDa),[3] turnip (27 kDa),[10] cabbage F-IA (40 kDa),[11] cabbage F-IB (43 kDa),[12] and Japanese radish root (46

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Table 1. Purification of the Cauliflower Enzyme

	PhO					POD				
enzyme	volume (mL)	total activity (unit)	total protein (mg)	specific activity (unit/mg)	purification (fold)	recovery (%)	total activity (unit)	specific activity (unit/mg)	purification (fold)	recovery (%)
crude extract	1890	228135 ± 4441	17614 ± 527.8	13 ± 1	1 ± 0.0	100.0 ± 0.0	369968 ± 2741	21 ± 1	1.0 ± 0.0	100.0 ± 0.0
crude enzyme	99	39594 ± 371	865 ± 13.1	46 ± 1	4 ± 0.1	17.4 ± 0.4	100980 ± 1648	117 ± 3	5.6 ± 0.1	27.3 ± 0.5
DEAE-Toyopearl 650-M	312	33073 ± 953	50 ± 2.7	662 ± 50	51 ± 4.1	14.5 ± 0.5	40850 ± 1894	817 ± 55	38.9 ± 1.8	11.0 ± 0.5
CM-Sephadex C-50	347	28258 ± 716	33 ± 2.0	853 ± 30	66 ± 3.5	12.4 ± 0.5	33934 ± 2408	1024 ± 37	48.8 ± 0.4	9.2 ± 0.7
butyl-Toyopearl 650-M	495	22579 ± 240	15 ± 0.0	1443 ± 16	111 ± 4.2	9.9 ± 0.3	28256 ± 913	1806 ± 59	86.0 ± 5.3	7.6 ± 0.2
Toyopearl HW 55-s	146	18448 ± 1063	5 ± 0.2	3656 ± 206	282 ± 16.5	8.1 ± 0.5	11698 ± 433	2318 ± 64	110.4 ± 6.2	3.2 ± 0.1

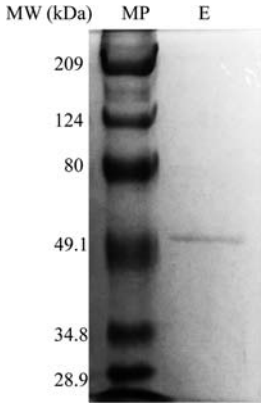

Figure 2. SDS–PAGE of the purified cauliflower enzyme. MW, molecular weight; MP, marker protein; E, enzyme.

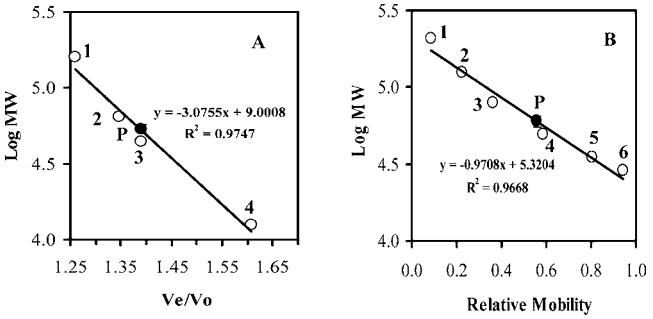

Figure 3. Molecular weight estimation of the cauliflower enzyme by gel filtration on (A) Toyopearl HW 55-s and (B) SDS–PAGE. (A) Vo, void volume of the column; Ve, elution volume of the substance; MW, molecular weight in kilodaltons; 1, α-globulin (160 kDa); 2, serum albumin (bovine, 65 kDa); 3, ovalbumin (chicken egg, 44 kDa); 4, cytchrome c (12.4 kDa); and P, purified enzyme. (B) MW, molecular weight in kilodaltons; 1, myosin (209 kDa); 2, β-galactosidase (124 kDa); 3, serum albumin (bovine, 80 kDa); 4, ovalbumin (chicken egg, 49.1 kDa); 5, carbonic anhydrase (34.8 kDa); 6, soybean trypsin inhibitor (28.9 kDa); and P, purified enzyme.

kDa),[14] while it was similar to the molecular weights of PPO of butter lettuce (60 kDa),[21] Japanese pear (56 kDa),[22] and broccoli florest (57 kDa).[23]

As shown in Table 2, the purified cauliflower PPO oxidized 1,3,5-tryhdroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid, but did not oxidize o-diphenols. A similar substrate specificity of cauliflower PPO had been found in the purified enzymes of Satsuma mandarin,[9] turnip,[10]

Table 2. Substrate Specificities of the Cauliflower PPO

substrates	specific activity (unit/mg)
phloroglucinol[a]	3656 ± 206
phloroglucinol carboxylic acid[a]	1926 ± 45
pyrogallol[b]	0 ± 0
gallic acid[b]	0 ± 0
catechol[b]	0 ± 0
chlorogenic acid[b]	0 ± 0
D,L-DOPA[b]	0 ± 0
dopamine[b]	0 ± 0
resorcinol[b]	0 ± 0

[a]Measured by the spectrophotometric method based on the difference in spectra.[17] [b]Measured by the colorimetric method.[4]

cabbage F-IA,[11] and cabbage F-IB.[12] In addition, the substrate specificity of the cauliflower enzyme differed from the specificity of the PPOs of Japanese radish root,[14] soybean,[15] and edible burdock,[24] which oxidized both 1,3,5-trihydroxybenzenes and 1,2,3-trihydroxybenzenes but did not oxidize *o*-diphenols. These results indicate that the cauliflower PPO is a group of a new type of PPO that is "phloroglucinol oxidase (PhO)".

As shown in Figure 4, the Michaelis constant (K_m) value of phloroglucinol oxidation of the purified cauliflower enzyme was

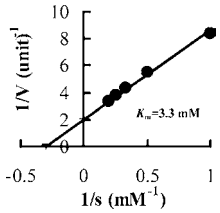

Figure 4. Lineweaver–Burk plots of phloroglucinol oxidation by the cauliflower enzyme.

3.3 mM. This K_m value was larger than the purified turnip PhO (0.67 mM)[10] but smaller than the cabbage PhO F-IA (6.4 mM)[11] and the cabbage PhO F-IB (8.5 mM).[12] The value of K_m indicated the affinity of the enzyme for enzyme–substrate complex formation. The K_m value of the cauliflower enzyme was larger than turnip PhO but smaller than cabbage PhO. It was indicated that cauliflower PhO has the greater affinity compared to cabbage PhO, but it reverses to turnip PhO.

The purified enzyme solution of the cauliflower showed a red color with absorption maxima at 405, 490, and 630 nm. Similar results were found in the purified enzymes of Satsuma mandarin,[9] turnip,[10] and cabbage,[11,12] which contained iron and had POD activity. As shown in Figure 1, the highest activity of PhO and POD of cauliflower was eluted in a similar fashion at the final step of purification.

The purified enzyme gave a single band of protein on SDS–PAGE (Figure 2). This result shows that the purified cauliflower enzyme has a dual activity of PhO and POD, just like the enzymes from Satsuma mandarin,[9] turnip,[10] and cabbage.[11,12] At the final step of purification, the POD activity of the cauliflower enzyme increased to 110 ± 6.2-fold with a recovery rate of 3.2 ± 0.1% (Table 1).

As shown in Figure 5A, the effect of pH on the activity of PhO and POD of purified cauliflower was greatly affected by pH. The optimum pH of PhO and POD of cauliflower was 8.0 and 4.0, respectively. The pH-dependent activity of PhO and

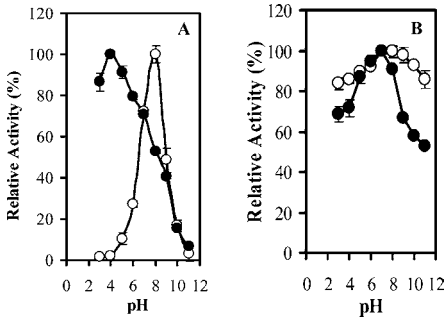

Figure 5. Effect of the pH on the (A) activity and (B) stability of the cauliflower enzyme: (○) PhO activity and (●) POD activity.

POD had also been found in the enzymes of Satsuma mandarin,[9] turnip,[10] and cabbage.[11,12] The optimum pH of cauliflower PhO was almost the same as that of turnip,[10] cabbage F-IA,[11] cabbage F-IB, and cabbage F-II;[12] the optima of these enzymes were in the range of pH 7.4–7.6. The optimum pH of cauliflower POD was close to that of cauliflower buds[16] with guaiacol as the substrate, broccoli,[25] and turnip[26] but different from that of cabbage F-IA (pH 6.4)[11] and cabbage F-II (pH 6.7).[12]

As shown in Figure 5B, the activity of PhO of the cauliflower enzyme was more stable than POD in the wide range of pH. Over 80% of PhO activity remained in the pH range of 3.0–11.0. However, the POD activity was stable at pH 5.0–8.0 and declined rapidly at the pH below 8.0 after 20 h of incubation at 5 °C. The stability of PhO and POD of cauliflower was slightly different from that of cabbage F-IA enzyme[11] in the range of pH 5.0–11.0. Gülçin and Köksal found the pH stability of cauliflower buds[16] using several substrates in the range of pH 8.0–9.0. In the present result, close pH stability was found from that of cauliflower buds using guaiacol as a substrate that is pH 8.5.

Figure 6 shows the effect of the temperature on the activity and stability of PhO and POD of the purified cauliflower

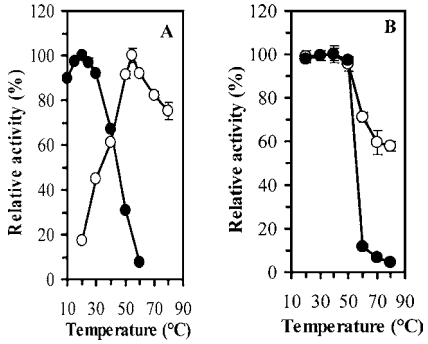

Figure 6. Effect of the temperature on the (A) activity and (B) stability of the cauliflower enzyme: (○) PhO activity and (●) POD activity.

enzyme. The optimum temperature of PhO and POD was 55 and 20 °C, respectively (Figure 6A). The optimum temperature of cauliflower PhO was almost the same as that of soybean (50 °C).[15] However, it was different from that of banana peel (30 °C)[5] and cabbage F-IA (40 °C).[11] The optimum temperature of cauliflower POD was different from that of cabbage F-IA (45 °C)[11] and slightly different from that of Turkish black radish (30 °C).[27]

Very high thermal stability of PhO had been found in the PhOs of Satsuma mandarin,[9] turnip,[10] and cabbage.[11,12] Contrarily, the purified enzyme of cauliflower showed a relatively low thermal stability. The activity of PhO and POD was stable at the temperature range of 20–50 °C. At the higher temperature than 50 °C, the activity of POD was almost lost. Contrarily, PhO was more heat-stable, and 60% of its activity remained after heating at 80 °C for 10 min (Figure 6B). The POD activity was close to that found by Rayan et al.,[13] which showed that the heat inactivation kinetics for POD cauliflower was at a temperature range of 65–85 °C. It indicated that the POD activity will be inactivated at a temperature above 65 °C.

Table 3 shows the effect of 13 kinds of compounds on the activity of PhO and POD of purified cauliflower enzyme. Sodium diethyldithiocarbamate inhibited PhO (IC_{50} = 0.64 and

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

	relative activity (%)			
	PhO		POD	
compounds	1 mM[a]	10 mM[a]	1 mM[a]	10 mM[a]
none	100 ± 0.39	100 ± 0.39	100 ± 1.38	100 ± 1.38
$NaS_2CN(C_2H_5)_2$[b]	12 ± 1.07	11 ± 0.79	91 ± 4.66	14 ± 0.19
KCN	94 ± 0.73	40 ± 2.94	1 ± 0.09	0 ± 0.01
EDTA	69 ± 6.41	68 ± 7.58	117 ± 0.47	119 ± 12.72
NaF	101 ± 1.01	102 ± 4.01	113 ± 11.08	122 ± 1.93
NaCl	106 ± 0.80	101 ± 0.39	107 ± 9.51	129 ± 3.94
$MnCl_2$	238 ± 5.13	278 ± 1.62	122 ± 9.42	127 ± 1.99
$CuSO_4$	35 ± 4.71	21 ± 1.50	99 ± 1.02	92 ± 0.97
$BaCl_2$	104 ± 0.33	128 ± 0.32	98 ± 1.08	94 ± 0.56
$ZnSO_4$	114 ± 1.17	123 ± 1.11	98 ± 0.47	91 ± 1.24
L-ascorbic acid	95 ± 1.60	0 ± 0.31	103 ± 0.97	0 ± 0.36
chlorogenic acid	18 ± 0.40	6 ± 0.66	16 ± 0.62	0 ± 0.14
resorcinol	109 ± 2.08	97 ± 1.33	108 ± 0.92	94 ± 0.76
hydroquinone	73 ± 3.24	46 ± 0.69	23 ± 0.59	1 ± 0.05

[a]Final concentration of compound. [b]Sodium diethyldithiocarbamate.

Table 4. Inhibition of Some Compounds on PhO and POD Activities

	PhO			POD		
compounds	K_i (mM)[a]	IC_{50} (mM)	type of inhibition	K_i (mM)[a]	IC_{50} (mM)	type of inhibition
sodium diethldithiocarbamate	0.15 ± 0.01	0.64 ± 0.04	competitive	1.64 ± 0.03	6.61 ± 0.14	competitive
KCN	1.95 ± 0.13	7.88 ± 0.54	competitive	$2.9 \times 10^{-2} \pm 0.2 \times 10^{-2}$[b]	0.03 ± 0.002	competitive
$CuSO_4$	0.16 ± 0.01	0.56 ± 0.05	noncompetitive	not measured[c]		
L-ascorbic acid	0.88 ± 0.02	3.55 ± 0.07	competitive	0.91 ± 0.020	3.67 ± 0.660	competitive
chlorogenic acid	0.19 ± 0.08	0.25 ± 0.10	non competitive	0.06 ± 0.002	0.24 ± 0.008	competitive
hydroquinone	1.93 ± 0.10	7.76 ± 0.41	competitive	0.16 ± 0.001	0.64 ± 0.005	competitive

[a]Final concentration of compound (10 mM). [b]Final concentration of compound (1.0 mM). [c]Slightly inhibited the POD activity.

K_i = 0.15) and POD (IC_{50} = 6.61 and K_i = 1.64) activities of the enzyme competitively. The inhibition rate for PhO was higher than that for POD at 1 mM. Competitive inhibitor of sodium diethyldithiocarbamate was also found in the PPO of nettle ($K_i = 1.79 \times 10^{-9}$).[16] KCN completely inhibited POD activity (IC_{50} = 0.03 and K_i = 29 μM) and also strongly inhibited PhO activity at 10 mM (IC_{50} = 7.88 and K_i = 1.95 mM) (Table 4). KCN inhibited both PhO and POD activities at 1 mM. These results indicate that the purified enzyme is metallic enzyme and the metal seems to be iron, as mentioned previously. L-Ascorbic acid competitively inhibited the activity of PhO and POD at 10 mM but not at 1 mM. The IC_{50} values for PhO and POD activities were 3.55 ± 0.07 and 3.67 ± 0.66 mM, respectively. $CuSO_4$ noncompetitively inhibited PhO activity (IC_{50} = 0.56 ± 0.05) and slightly inhibited POD activity at 1 and 10 mM. Similar results were obtained for the PhOs from cabbage F-IA,[11] cabbage F-IB, and cabbage F-II.[12] Ethylenediaminetetraacetic acid (EDTA) inhibited PhO and slightly activated POD of cauliflower. $BaCl_2$ and $ZnSO_4$ showed a weak activation of PhO and a weak inhibition of POD. However, the strong activation of PhO was induced by $MnCl_2$. These results were similar from the PhOs of turnip[10] and cabbage,[11,12] which showed a remarkable activation by $MnCl_2$. The activity of PhO and POD of cauliflower was markedly inhibited by chlorogenic acid (*o*-diphenols) and hydroquinone (*p*-diphenol) in 1 and 10 mM, just like the enzymes of Satsuma mandarin[9] and cabbage.[11,12] Chlorogenic acid showed competitive inhibition for PhO activity and noncompetitive inhibition for POD activity. Hydroquinone competitively inhibited both PhO and POD activities of the purified enzyme. It is interesting that the type of inhibition of chlorogenic acid to the cauliflower enzyme for both PhO and POD activities was different. Even though both activities appeared in the same peak, chlorogenic acid inhibited the PhO activity with a noncompetitive type and inhibited the POD activity with a competitive type. It is indicated that both PhO and POD have separate active sites.

In conclusion, the PhO of cauliflower was purified to a homogeneous state on SDS−PAGE. The purified cauliflower enzyme oxidized 1,3,5-trihdroxybenzenes but did not oxidize 1,2,3-trihydroxybenzenes and *o*-diphenols. These results may indicate that this enzyme is a group of a new type of PPO, "phloroglucinol oxidase (PhO)", which is similar to the enzymes of Satsuma mandarin,[9] turnip,[10] and cabbage.[11,12] The purified cauliflower enzyme showed a dual activity of PhO and POD. The activity of PhO and POD was affected in a different manner by pH, temperature, various compounds, and type of inhibition tested. On the basis of these results, we speculate that the purified cauliflower enzyme has separate active sites for PhO and POD. The results of biochemical properties of the cauliflower enzyme were also beneficial in an attempt to inhibit or control PhO and POD activities in cauliflower during storage and processing.

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Notes

The authors declare no competing financial interest.

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