ダイコン (Raphanus sativus L.) のポリフェノール酸化酵素の精製と性質

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Purification and Characterization of Polyphenol Oxidase from Japanese Radish (*Raphanus sativus* L.) Root

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Polyphenol oxidase (PPO) was purified from the Japanese radish root by ammonium sulfate fractionation, ion exchange chromatography, hydrophobic chromatography, and gel filtration using phloroglucinol as a substrate. The enzyme was purified about 192-fold with a recovery rate of 15%. The purified enzyme appeared as a single band on SDS-PAGE. The molecular weight of the purified PPO was estimated to be about 44 kDa by gel filtration and 45.7 kDa by SDS-PAGE. The purified enzyme quickly oxidized phloroglucinol (1, 3, 5-trihydroxybenzene) with a K_m of 2 mM. The enzyme also oxidized 1, 2, 3-trihydroxybenzenes, such as pyrogallol and gallic acid; however, it did not oxidize *o*-diphenols, such as chlorogenic acid and dopamine. Peroxidase (POD) activity was also present in the purified enzyme preparation with the final preparation having a purification and recovery rate of 259-fold and 20%, respectively. The optimum pH for the PPO and POD activities was 8.0 and 5.0, respectively, and the measured activities were stable at 5℃ for 20 h in the pH ranges of 3.0～10.0 and 3.0～11.0, respectively. Both enzyme activities were stable up to 50℃ after heat treatment for 10 min and were strongly inhibited by treatment with sodium diethyldithiocarbamate, potassium cyanide, L-ascorbic acid, chlorogenic acid, and hydroquinone at a final concentration of 10 mM.

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Key words : *Japanese radish, polyphenol oxidase, phloroglucinol oxidase, peroxidase, characterization*
ダイコン，ポリフェノール酸化酵素，フロログルシノール酸化酵素，パーオキシダーゼ，精製と性質

The undesirable browning of damaged tissues in fruits and vegetables occurs by the enzymatic oxidation of polyphenols. Such oxidation is mainly caused by polyphenol oxidase (EC 1.10.3.1 ; *o*-diphenol : oxygen oxidoreductase, PPO). Because browning can decrease the marketability of variety of fruits and vegetables, many studies have investigated PPO with the goal of preventing this discoloration[1)~9)]. PPOs that originate from distinct plant sources display different substrate specificities. Most PPOs oxidize *o*-diphenols, such as catechol, chlorogenic acid, dopamine, DL-dopa, and 4-methylcatechol. For example, purified PPOs from edible burdock[5)], garland chrysanthemum[6)], and apple[7)] are known to strongly oxidize chlorogenic acid, while banana PPO[10),11)] strongly oxidizes dopamine. In contrast, purified PPOs of edible burdock[12)] and soybean[13)] oxidize pyrogallol (1, 2, 3-trihydroxybenzene) and phloroglucinol (1, 3, 5-trihydroxybenzene) but do not oxidize *o*-diphenols. However, a novel type of PPO, which only oxidizes 1, 3, 5-trihydroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid, was found by our laboratory in Satsuma mandarin[14)], cabbage[15),16)], and turnip[17)]. These purified phloroglucinol-oxidizing PPOs (PhOs) also have strong peroxidase (EC 1.11.1.7 ; POD) activities. While purified soybean PPO[13)] has been shown to have dual PPO and POD activities, edible burdock PPO[5)] was not found to have POD activity. PhO activity was found in crude extracts of the Japanese radish (*Raphanus sativus* L.) root, which is considered a cruciferous vegetable similar to cabbage and turnip. Interestingly, PPO that was purified from the Japanese radish root was found to

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have dual PPO and POD activities, which is similar to that of soybean[13], cabbage[15,16], and turnip[17] PPOs ; however, a detailed characterization of Japanese radish root PPO has not previously been conducted. In the present study, Japanese radish root PPO was purified using phloroglucinol as a substrate, and the properties of the purified enzyme were investigated.

Materials and Methods

1. Materials

Fresh Japanese radish (*Raphanus sativus* L. cv. Aokubi soufuto-L.) root was purchased from a local market in Saga City. DEAE-Toyopearl 650-M, Butyl Toyopearl 650-M, and Toyopearl HW 55-s were obtained from Tosoh Co., Tokyo, Japan. Other reagents were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

2. Measurement of enzyme activity

(1) PhO activity PhO activity was measured by a spectrophotometric method that was based on differences in spectra[18]. The reaction mixtures consisted of 0.5 mℓ of 20 mM aqueous phloroglucinol, 1.4 mℓ of 0.1 M phosphate buffer (pH 7.0), and 0.1 mℓ of each fraction obtained by chromatography. After incubation for 10 min at 30℃, 0.5 mℓ of the reaction mixture was removed and added to 4.5 mℓ of distilled water. The absorbance of the sample was then measured at 272 nm against an enzyme blank. One unit of 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.

(2) PPO activity PPO activity was measured by a colorimetric method[10,19,20]. The reaction mixtures consisted of 0.5 mℓ of 10 mM aqueous solution of various polyphenols, 4 mℓ of 0.1 M phosphate buffer (pH 7.0), and 0.5 mℓ of enzyme solution. After a 5-min incubation at 30℃, the increase in absorbance at 420 nm (ΔA_{420}) was measured. One unit of enzyme activity was defined as 0.1 ΔA_{420} per min (1-cm light path).

(3) POD activity POD activity was determined by a colorimetric method[15]. The reaction mixtures 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℃, the increase in absorbance at 470 nm (ΔA_{470}) was measured. One unit of enzyme activity was defined as 0.1 ΔA_{470} per min (1-cm light path).

3. Purification of the enzyme

All steps were carried out at 5℃. Whole roots from the Japanese radish (10 kg ; 9～10 radishes) were homogenized with a Japanese-style grater without using buffer. After filtration of the homogenate through a cotton cloth, the filtrate was centrifuged at 10,300 × *g* at 4℃ for 20 min. The supernatant was then brought to 80% saturation with ammonium sulfate. Precipitated protein was collected by centrifugation (10,300 × *g*), dissolved in a small volume of 0.01 M phosphate buffer (pH 7.0), and then dialyzed against the same buffer for 36 h with the four changes of the dialysis buffer. After another round of centrifugation (10,300 × *g*), the dialyzed solution was applied to a DEAE-Toyopearl 650-M column (4.5 × 15 cm) that was equilibrated with 0.01 M phosphate buffer (pH 7.0) and eluted with the same buffer. Fractions containing active PPO, which were not adsorbed onto the column, were pooled, brought to 1 M saturation with ammonium sulfate, and then applied to a Butyl-Toyopearl 650-M column (1.6 × 15 cm) that was equilibrated with 0.01 M phosphate buffer (pH 7.0) containing 1 M ammonium sulfate. The column was eluted using a linear gradient of ammonium sulfate (1 to 0 M ammonium sulfate in 0.01 M phosphate buffer, pH 7.0). Fraction containing PPO activity were pooled and dialyzed with 0.01 M phosphate buffer (pH 7.0). The dialyzed solution was concentrated using a membrane filter (Amicon YM-10, Millipore Japan Co., Tokyo, Japan), added to a Toyopearl HW 55-s column (1.6 × 80 cm) that was equilibrated with 0.1 M phosphate buffer, and then eluted using the same buffer. Fractions containing enzyme activity were collected and used for enzyme characterization.

4. Determination of protein

Protein content was determined using the method of HARTREE[21] with bovine serum albumin (BSA, fraction V ; Katayama Chemical, Industries Co., Ltd., Osaka, Japan) used as a standard. In chromatography, protein was expressed as absorbance at 280 nm.

5. Molecular weight determination

Molecular weight of the purified enzyme was determined by gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gel filtration was performed following the method of ANDREWS[22] with the use of α-globulin (160 kDa), serum albumin (bovine ; 65 kDa), ovalbumin

(chicken egg ; 44 kDa), and cytochrome c (12.4 kDa) as marker proteins. SDS-PAGE was carried out as described by WEBER and OSBORN[23] with the use of myosin (209 kDa), β-galactosidase (124 kDa), serum albumin (bovine ; 80 kDa), ovalbumin (chicken egg ; 49.1 kDa), carbonic anhydrase (34.8 kDa), soybean trypsin inhibitor (28.9 kDa), and lysozyme (20.6 kDa) as marker proteins.

Results and Discussion

(1) Purification of the enzyme Fig. 1 shows a typical elution pattern of PPO on a gel filtration column. Enzyme activity eluted in one peak, and the peak fractions from several columns were pooled as purified enzyme. A typical stepwise purification of PPO is shown in Table 1. After the final purification steps, the enzyme preparation contained a specific activity that was increased 192-fold compared to that of the initial homogenate with a recovery rate of 15%.

(2) Characterization of the purified enzyme

The purified enzyme appeared as a single band on SDS-PAGE (Fig. 2). As shown in Fig. 3, the molecular weight of PPO was estimated to be about 44 kDa by gel filtration and 45.7 kDa by SDS-PAGE. These results indicate that the purified enzyme is monomeric. While the molecular weight of the purified Japanese radish root PPO was similar to soybean PPO (47 kDa)[13] and leaf lettuce PPO (46 kDa)[24], it differed from those of edible burdock (40 kDa)[5], cabbage F-IA (40 kDa)[15], cabbage F-IB (43 kDa)[16], turnip (27 kDa)[17], Japanese pear (56 kDa)[20], broccoli (57 kDa)[25], and Turkish black radish (66 kDa)[26].

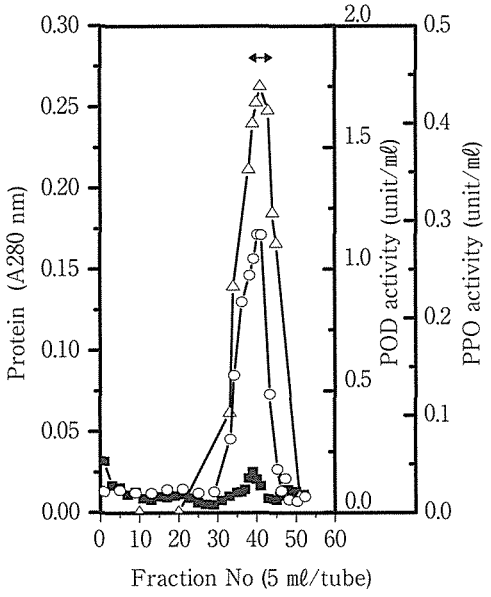

Fig. 1 Elution pattern of the enzyme from the Toyopearl HW 55-s column

(↔) fraction pooled ; (○) PPO activity ; (■) protein ; (△) POD activity.

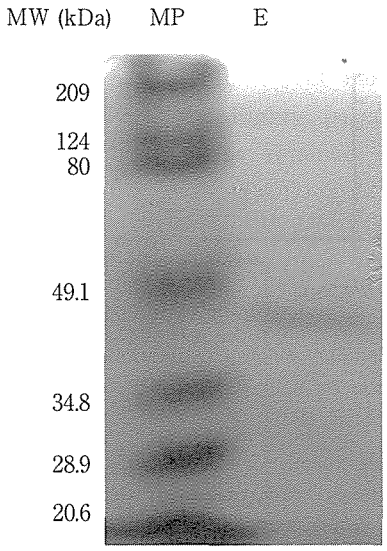

Fig. 2 SDS-PAGE of the purified enzyme

MW, molecular weight; MP, marker protein; E, Enzyme.

Table 1 Purification of the Japanese radish root enzyme

Enzyme	Phloroglucinol Oxidase (PhO)						Peroxidase (POD)			
	Volume (mℓ)	Total Activity (Units)	Total Protein (mg)	Specific Activity (Unit/mg)	Purification (fold)	Recovery (%)	Total Activity (Units)	Specific Activity (Unit/mg)	Purification (fold)	Recovery (%)
Crude Extract	6,890	689,345	35,812	19	1.0	100	1,302,210	36	1.0	100
Crude Enzyme	490	302,967	2,379	127	6.6	44	514,338	216	6.0	40
DEAE-Toyopearl 650-M	1,225	379,922	266	1,428	74.2	55	456,404	1,716	47.2	35
BUTYL-Toyopearl 650-M	931	143,421	50	2,875	149.4	21	328,696	6,589	181.2	25
Toyopearl HW 55-s	1,250	103,167	28	3,694	192.0	15	262,938	9,415	259.0	20

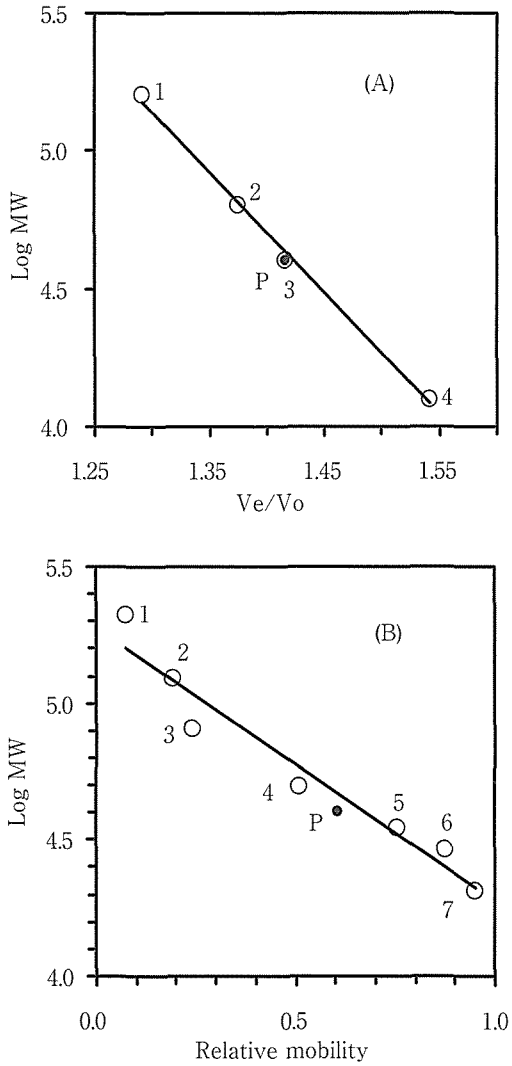

Fig. 3 Molecular weight estimation of the enzyme by gel filtration on a Toyopearl HW 55-s column (A) and by SDS-PAGE (B)

(A) Vo, void volume of the column ; Ve, elution volume of the substance ; MW, molecular weight : 1. α-globulin (160 kDa), 2. serum albumin (bovine ; 65 kDa), 3. ovalbumin (chicken egg ; 44 kDa), and 4. cytochrome c (12.4 kDa) ; P, purified enzyme.

(B) MW, molecular weight in kDa : 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 7. lysozyme (20.6 kDa) ; P, purified enzyme.

Table 2 Substrate specificities of Japanese radish root PPO

Substrate	Specific Activity (Unit/mg)
Phloroglucinol[1]	3,694
Phloroglucinol carboxylic acid[1]	1,692
Pyrogallol[2]	91
Gallic acid[2]	16
Catechol[2]	0
Chlorogenic acid[2]	0
DL-Dopa[2]	0
Dopamine[2]	0
Resorcinol[2]	0

[1]Measured by a spectrophotometric method based on differences in spectra[18]
[2]Measured by a colorimetric method[10),19),20)]

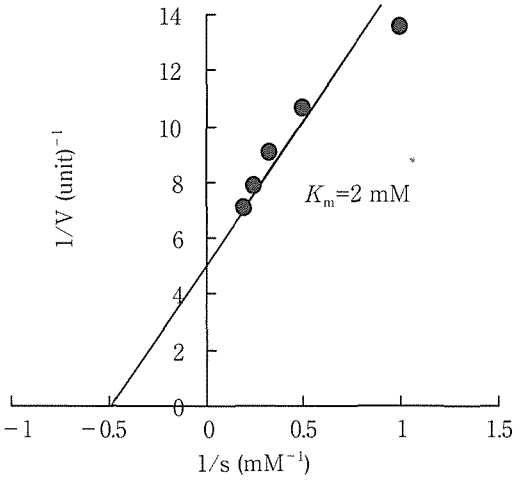

Fig. 4 Lineweaver-Burk plots of phloroglucinol oxidation by the enzyme

Most PPOs oxidize *o*-diphenols, such as catechol, chlorogenic acid, dopamine, DL-dopa, and 4-methylcatechol. PPOs purified from edible burdock[5], garland chrysanthemum[6], and apple[7] strongly oxidize chlorogenic acid, while banana PPO[10),11)] strongly oxidizes dopamine. As shown in Table 2, purified Japanese radish root PPO strongly oxidizes 1, 3, 5-tryhdroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid. The Michaelis constant (K_m) of the enzyme for the oxidation of phloroglucinol was 2 mM (Fig. 4). This value differed from that of other plant PPOs, specifically, Satsuma mandarin PPO (0.67 mM)[14], cabbage PPO F-IA (6.4 mM)[15], and cabbage PPO F-IB (8.5 mM)[16]. PPO purified from the Japanese radish root also oxidized 1, 2, 3-trihydroxybenzenes, such as pyrogallol and gallic acid ; however, it did not oxidize *o*-diphenols, such as catechol, chlorogenic acid, and dopamine, or *p*-diphenols, such as resorcinol. This enzyme was found to share a similar substrate specificity as that of edible burdock PPO[12] and soybean PPO[13]. However, the substrate specificities of these three enzymes were different from the PPOs of Satsuma mandarin[14],

cabbage[15),16)], and turnip[17)], which only oxidized 1, 3, 5-trihydroxybenzenes.

The purified Japanese radish root enzyme preparation was red in color with an absorption maxima at 405, 490, and 630 nm, which was similar to that of soybean PPO[13)]. Similar results were obtained using purified PPOs of Satsuma mandarin[14)], cabbage[15),16)], and turnip[17)], which contain iron. Soybean, Satsuma mandarin, cabbage, and turnip enzymes had dual PPO and POD activities. As shown in Fig. 1, activities of both PPO and POD were detected in the same peak fraction with the final purification of the Japanese radish root enzyme. Furthermore, the purified enzyme was visualized as a single protein band on SDS-PAGE (Fig. 2). Therefore, we reasoned that the Japanese radish root enzyme has dual PPO and POD activities, similar to the soybean[13)], cabbage[15),16)], and turnip[17)] enzymes. The final preparation of the purified enzyme showed a 259-fold increase in POD activity and had a recovery rate of 20% for POD (Table 1). Further characterization of the PPO and POD activities of the enzymatic preparation was conducted. The effects of pH on the activity and stability of both PPO and POD activities of the purified enzyme are shown in Fig. 5. The optimal pH for PPO and POD activities was 8.0 and 5.0, respectively (Fig. 5 A). The effects of changes in the pH on PPO and POD activities of the PPO enzyme have also been demonstrated for PPOs in soybean[13)], Satsuma mandarin[14)], cabbage[15),16)], and turnip[17)]. The optimal pH for the activity of the Japanese radish root PPO enzyme was similar to PPOs purified from soybean[13)], cabbage F-IA[15)], cabbage F-IB, cabbage F-II[16)], and turnip[17)], which were shown to function best in the optimal pH range of 7.4～7.6. However, the optimal pH for our purified Japanese radish root PPO differed from that of butter lettuce PPO (pH 5.5)[8)]and vanilla bean PPO (pH 3.0)[9)]. The optimal pH for POD activity in our purified Japanese radish root enzymatic preparation was also similar to that of Satsuma mandarin[14)], and turnip[17)], but it differed from cabbage enzyme F-IA (pH 6.4)[15)], and F-II (pH 6.7)[16)].

As shown in Fig. 5 B, both PPO and POD activities of the Japanese radish root enzyme were stable in wide pH ranges. Eighty percent of the PPO and POD activities was retained in the pH

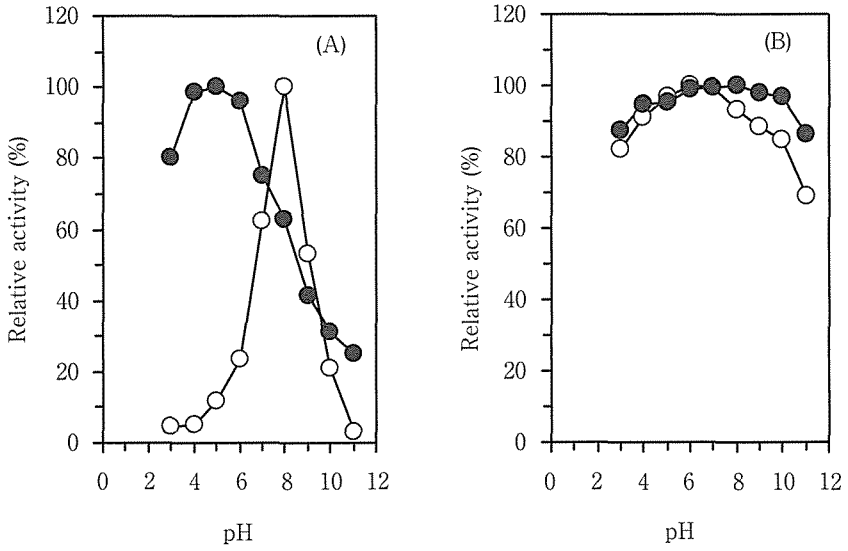

Fig. 5 Effects of pH on the activity (A) and stability (B) of the enzyme

(○) PPO activity ; (●) POD activity

(A) PPO and POD activities were measured at 30℃ in McIlvaine buffer in the pH range 3.0～8.0 and in Atkins-Pantin buffer in the pH range 9.0～11.0. Activity was expressed as a percentage of the maximum activity level.

(B) The enzyme was pre-incubated in McIlvaine buffer at different pH values, ranging from 3.0～8.0 or in Atkins-Pantin buffer at different pH values, ranging from 9.0～11.0 at 5℃ for 20 h. Residual PPO and POD activities were measured under standard conditions (PPO: pH 7.0, 30℃ and POD: pH 6.0, 30℃). Activity was expressed as a percentage of the maximum activity level.

ranges 3.0～10.0 and pH 3.0～11.0, respectively. The ranges of pH stability for both PPO and POD activities differed slightly from those of cabbage F-IA PPO[15], in which both activities were stable in the range of pH 5.0～11.0. Similar results were found for Satsuma mandarin PPO[14] and turnip PPO[17].

The PPO enzyme from Satsuma mandarin[14], cabbage[15),16)], and turnip[17] had a very high thermal stability. Fig. 6 shows the thermal stability of the purified Japanese radish root PPO enzyme. After heating at 80℃ for 10 min, only 20% of the PPO activity remained ; however, POD activity was almost completely lost. The thermal stability of this preparation of PPO enzyme was lower than that of the above enzymes (specifically, that of Satsuma mandarin[14], cabbage[15),16)], and turnip[17]. In contrast, the soybean enzyme[13] had a similar thermal stability of PPO and POD activities as the Japanese radish root enzyme.

Table 3 shows the effects of different compounds on the PPO and POD activities of the purified enzyme. Both the PPO and POD activities were markedly inhibited by sodium diethyldithiocarbamate and potassium cyanide when used at a 10 mM final concentration. Additionally, L-ascorbic acid inhibited both PPO and POD activities at 10 mM but not at 1 mM. $CuSO_4$ inhibited PPO activity, but did not inhibit POD activity. Remarkably, $MnCl_2$ was found to activate PPO in edible burdock[12], cabbage[15),16)], and turnip[17] ; however, little activation was found for Japanese radish root PPO. Both PPO and POD activities were markedly inhibited by chlorogenic acid (an *o*-diphenol) and hydroquinone (a *p*-diphenol) which is similar to studies using purified from Satsuma mandarin[14] and cabbage[15),16)].

In conclusion, Japanese radish root PPO was purified using phloroglucinol as a substrate. The purified enzyme quickly oxidized 1, 3, 5-trihydroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid, and 1, 2, 3-trihydroxybenzenes, such as pyrogallol ; however, it did not oxidize *o*-diphenols, such as chlorogenic acid and dopamine, which was similar to edible burdock PPO[12] and soybean PPO[13]. Furthermore, the substrate specificities of these enzymes differed from those of Satsuma mandarin PPO[14], cabbage PPO[15),16)], and turnip PPO[17], which only oxidized 1, 3, 5-trihydroxybenzene. POD activity was also found in the purified Japanese radish root PPO. Due to the different effects of pH, temperature, and various compounds on PPO and POD activities of the purified Japanese radish root enzyme, it probable that this enzyme has separate active sites for PPO and POD activities, and this may also be the case

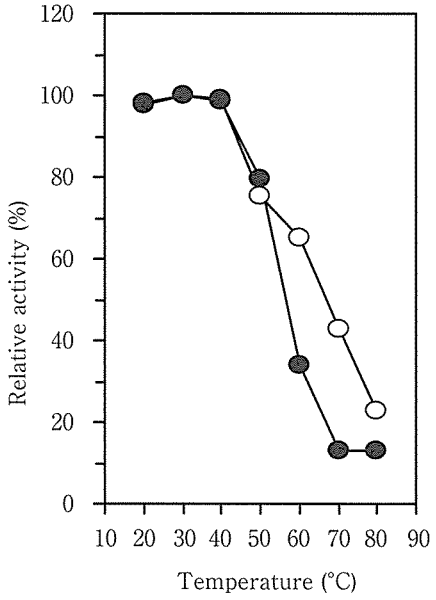

Fig. 6 Effects of temperature on the stability of the enzyme

(○) PPO activity ; (●) POD activity.
The enzyme preparation was heated at temperatures ranging from 20℃～80℃ for 10 min. Residual PPO and POD activities were measured under standard conditions (PPO : pH 7.0, 30℃ and POD : pH 6.0, 30℃). Activity was expressed as a percentage of the maximum activity level.

Table 3 Effects of various compounds on PPO and POD activities

Compounds	Relative Activity (%)			
	PPO activity		POD activity	
	1 mM[a]	10 mM[a]	1 mM[a]	10 mM[a]
None	100	100	100	100
Sodium diethyldithiocarbamate	11	0	89	33
KCN	87	32	0	0
EDTA	98	94	94	92
NaF	105	110	103	83
NaCl	103	100	97	92
$MnCl_2$	128	132	93	90
$CuSO_4$	37	13	111	96
$BaCl_2$	115	97	108	119
$ZnSO_4$	109	107	105	103
L-ascorbic acid	109	0	97	1
Chlorogenic acid	21	16	48	1
Resorcinol	116	104	104	96
Hydroquinone	28	26	33	1

[a]Final concentration of compound

for soybean[13], cabbage[15),16)], and turnip[17] PPOs.

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ダイコン（*Raphanus sativus* L.）のポリフェノール酸化酵素の精製と性質

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ダイコンのポリフェノール酸化酵素（PPO）をフロログルシノールを基質として，硫安分画，イオン交換クロマト，疏水クロマトおよびゲル濾過により精製した。本酵素は192倍に精製され，回収率は15%であった。精製酵素はSDS-PAGEで単一バンドを呈した。本酵素の分子量はゲル濾過で44 kDa，SDS-PAGEで45.7 kDaと推定された。本酵素は１，３，５-トリヒドロキシベンゼンであるフロログルシノールを強く酸化し，それに対するK_m値は２ mMであった。本酵素は同時に１，２，３-トリヒドロキシベンゼンであるピロガロールおよび没食子酸を酸化したが，クロロゲン酸やドーパミンのような*o*-ジフェノール類は酸化しなかった。本酵素はパーオキシダーゼ（POD）活性を同時に有し，PODとしては259倍に精製され，回収率は20%であった。本酵素のPPOおよびPOD活性の最適pHはそれぞれ８及び５であり，PPOはpH 3.0～10.0の範囲で，PODは3.0～11.0の範囲で５℃，20時間安定であった。熱安定性においては両活性とも50℃まで安定であった。また，両活性とも終濃度10 mMにおいて，ジエチルジチオカルバミン酸ナトリウム，シアン化カリウムのような金属酵素阻害剤，還元剤であるL-アスコルビン酸およびジフェノール類であるクロロゲン酸やヒドロキノンにより強く阻害された。

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