# Partial Purification of Polyphenol Oxidase from Chinese Cabbage *Brassica rapa* L.

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Polyphenol oxidase (PPO) was purified and characterized from Chinese cabbage by ammonium sulfate precipitation and DEAE-Toyopearl 650M column chromatography. Substrate staining of the crude protein extract showed the presence of three isozymic forms of this enzyme. The molecular weight of the purified enzyme was estimated to be ~65 kDa by gel filtration on Toyopearl HW-55F. On SDS-PAGE analysis, this enzyme was composed of a subunit molecular weight of 65 kDa. The optimum pH was 5.0, and this enzyme was stable at pH 6.0 but was unstable below pH 4.0 or above pH 7.0. The optimum temperature was 40 °C. Heat inactivation studies showed temperatures >40 °C resulted in loss of enzyme activity. PPO showed activity to catechol, pyrogallol, and dopamine ($K_m$ and $V_{max}$ values were 682.5 mM and 67.6 OD/min for catechol, 15.4 mM and 14.1 OD/min for pyrogallol, and 62.0 mM and 14.9 OD/min for dopamine, respectively). The most effective inhibitor was 2-mercaptoethanol, followed in decreasing order by ascorbic acid, glutathione, and L-cysteine. The enzyme activity of the preparation was maintained for 2 days at 4 °C but showed a sudden decreased after 3 days.

**Keywords:** *Polyphenol oxidase; PPO; Chinese cabbage; partial purification; characterization; inhibitors*

## INTRODUCTION

Enzymatic browning occurs in vegetables and fruits after bruising or cutting or during storage. This results from oxidation of phenolic compounds to quinones by polyphenol oxidase (PPO), in the presence of oxygen (*1*). The quinones condense to form dark pigments (*2*). Enzymatic browning is also an economic problem for processors and consumers. PPO is a copper-containing enzyme and widely distributed in plants (*3*). It has been studied in several plant tissues such as apples (*4*−*7*), bananas (*8*−*10*), peaches (*11*), grapes (*12*, *13*), kiwis (*14*), pears (*15*, *16*), raspberries (*17*), strawberries (*18*), plums (*19*), herbs (*20*), spinach (*21*), broad beans (*22*, *23*), field beans (*24*), wild potatoes (*25*), Jerusalem artichokes (*26*, *27*), cabbages (*28*), and tea leaves (*29*, *30*). The enzyme catalyzes two different reactions involving molecular oxygen. The first is the hydroxylation of monophenols leading to *o*-diphenols (monophenol monooxygenase: EC 1.14.18.1). The second is the oxidation of *o*-diphenols to *o*-quinones (diphenol oxidase: EC 1.10.3.2). Many investigators have reported that PPOs from different plant tissues showed different substrate specificities and degrees of inhibition (*31*).

Chinese cabbage is used as a material for pickled vegetables, *tsukemono*, and it is consumed in Japan on a daily basis. When it is stored in a refrigerator, the plant develops unpleasant colors and flavors and loses nutrients when it brows. Therefore, it is necesassary to characterize the PPO to develop more effective methods for controlling browning in Chinese cabbage.

The objective of our study was to characterize the PPO from Chinese cabbage. This information will be useful in devising effective methods for inhibiting browning during storage.

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## MATERIALS AND METHODS

**Materials.** Bio-Beads SM-2 was obtained from Bio-Rad Laboratories (Tokyo, Japan). Catechol, tyrosine, pyrogallol, L-3,4-dihydroxyphenylalanine (L-DOPA), chlorogenic acid, and Triton X-100 were from Wako Pure Chemicals (Osaka, Japan). Other reagents were of analytical grade. Chinese cabbage *Brassica rapa* L. was purchased from the local wholesale market in Shimonoseki City, Yamaguchi Prefecture, Japan, and kept in a refrigerator until used.

**Assay of PPO Activity.** Enzyme activity for PPO was assayed in triplicate according to the method of Siddiq et al. (*19*). The standard reaction mixture consisted of 250 μL of 0.2 M sodium phosphate buffer (pH 6.0), 50 μL of 1.0 M catechol, and 50 μL of enzyme solution. The reaction was carried out at 30 °C for 5 min, and PPO activity was measured by monitoring the increase in absorbance at 420 nm. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001 per minute.

**Determination of Protein Concentration.** The protein concentration was measured according to the method of Lowry et al. (*32*) using bovine serum albumin as standard.

**Electrophoresis.** Two kinds of polyacrylamide gel electrophoresis (PAGE) were carried out: native PAGE was according to the method of Davis (*33*) and SDS-PAGE according to the method of Laemmli (*34*).

**Activity Staining.** After native PAGE, PPO activity was visualized with catechol as substrate following the method of Vanloon (*35*). The gel was equilibrated for 30 min at room temperature in 50 mM citrate−phosphate buffer (pH 5.0) and was then incubated for 30 min in 50 mM catechol in the same buffer under vigorous aeration. Black bands indicated sites of PPO activity.

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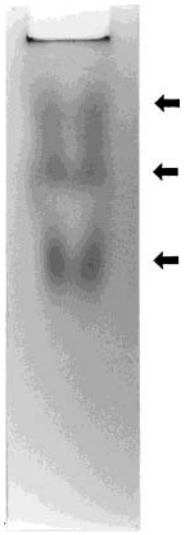

**Figure 1.** Substrate staining of PPO with catechol as substrate.

**Table 1. Partial Purification of PPO from Chinese Cabbage**

| step | total protein (mg) | total act. (units) | specific act. (units/mg) | purifn fold |
|---|---|---|---|---|
| crude | 526.0 | 56800 | 108.0 | 1 |
| 40−80% $(NH_4)_2SO_4$ | 167.3 | 32870 | 196.5 | 1.8 |
| DEAE-Toyopearl 650M | 5.4 | 5879 | 1088.7 | 10.1 |

## RESULTS AND DISCUSSION

**Partial Purification of PPO.** All extraction materials were maintained at low temperature (2−5 °C) to reduce enzymatic activity during extraction. For crude extract, 50 g of Chinese cabbage tissue was quickly cut into thin slices and extracted with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.5% (v/v) Triton X-100. After gentle stirring for 2 h, the homogenate was filtered with glass wool and the filtrate was centrifuged at 25000*g* for 1 h. Triton X-100 was removed from the supernatant using a Bio-Beads SM-2 column (1.3 × 6.0 cm; Bio-Rad Laboratories) previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0). After the column had been washed with the same buffer, the washings and eluate were combined and dialyzed against the same buffer. The dialyzed sample was fractionated with solid ammonium sulfate, and the precipitate from 40−80% saturation was dissolved in a minimal volume of the same buffer. After dialysis, the active fractions were applied to a DEAE-Toyopearl 650M column (1.0 × 5.0 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 6.0). The enzyme was not absorbed to this column. The eluate was used as the PPO enzyme source in the following experiments. The purification procedures are summarized in Table 1. Finally, PPO was purified up to 10.1-fold with a recovery rate of 10.4% as compared with crude extract.

**Isozyme Profile.** To elucidate the presence of isozymes in the crude extract, a native PAGE was performed, and the gel was stained with catechol. The crude enzyme solution revealed the presence of three bands for this activity (Figure 1). In contrast, Takeo and Baker (*29*) reported at least six forms of PPO activity with various isoelectric points found by isoelectric focusing in tea leaves. Sanderson reported a large number of bands in tea shoot tips by using the same technique (*36*). Park and Luh (*14*) reported four forms of PPO as isozymes in kiwis. Rivas and Whitaker (*16*) reported the existence of two types of PPO in Bartlett pears. Our result differed from these but agreed with those of Flurkey and Jen (*11*), who reported three types of PPO in peach. It is suggested that the number of PPO isozymes differs among species.

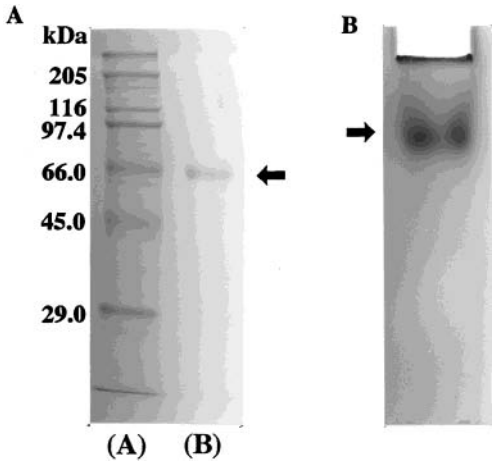


**Figure 2.** Purification of PPO: (A) SDS-PAGE of partially purified PPO (lane 1, molecular weight marker; lane 2, purified PPO); (B) non-denaturing PAGE substrate staining.

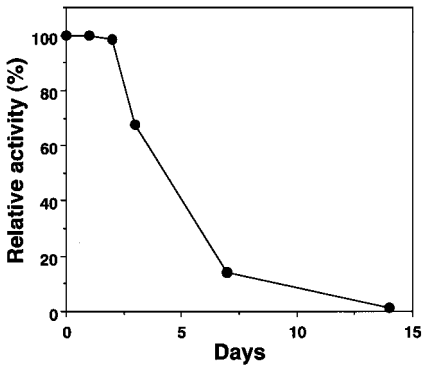


**Figure 3.** Effect of storage time on the activity of Chinese cabbage PPO.

**Molecular Weight Determination.** The molecular weight of PPO was ∼65 kDa when estimated by Toyopearl HW-55F gel filtration. On SDS-PAGE, the enzyme produced a single band of ∼65 kDa (Figure 2A). After native PAGE, the gel was stained with catechol. The final purified enzyme showed a single band (Figure 2B). These results indicate that the purified enzyme may be a monomer having a molecular weight of 65 kDa. The molecular weight of PPO from other species has been reported as follows: tea leaf, 72 kDa (*30*); sunflower seeds, 42 kDa (*37*); apple, 65 kDa (*5*, *6*); banana, 41 kDa (*10*) and 62 ± 2 kDa (*9*); cabbage, 39 kDa (*28*); and field bean seed, 120 ± 3 kDa (*24*). Our result indicates that the molecular weight of Chinese cabbage PPO was similar to those of tea leaf, apple, and banana but was different from those of sunflower seeds, banana, cabbage, and field bean seed.

**Effect of Storage Time on Enzyme Activity.** Enzyme solution was stored at 4 °C for 14 days, and the residual activity was measured. The result shows that activity was retained for 2 days but suddenly decreased after 3 days and was ∼15% for 7 days (Figure 3). Wissemann and Montgomery (*15*) reported that 25% of the enzyme activity was retained when during storage

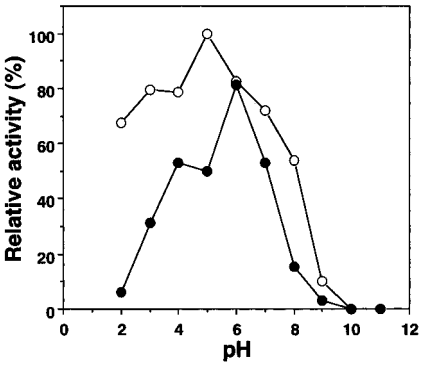


**Figure 4.** Optimum pH and stability curves of PPO. For each pH, activity was assayed at 30 °C as described under Materials and Methods and expressed as relative activity (○). The pH stability curve (●) represents the residual activity after a preincubation period of 1 day at the indicated pH.

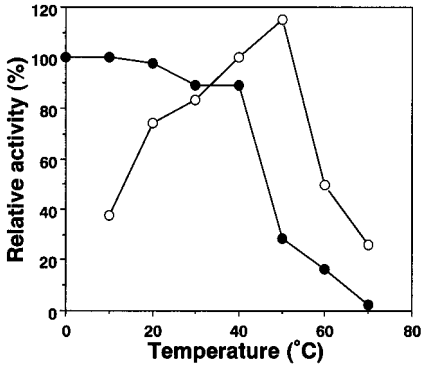


**Figure 5.** Effect of temperature on the activity and stability of PPO. Enzyme activity was assayed for each temperature after an incubation period of 5 min (○). For the thermal stability experiments (●), the enzyme was prewarmed at the indicated temperature for 60 min, cooled to 4 °C, and then the remaining activity was determined.

at 4 °C for 15 days. On the other hand, Kowalski et al. (*25*) reported that wild potato PPO was stable at −70 °C for several weeks, but it was stable at 4 °C for only 1 day.

**Effect of pH on Enzyme Activity.** The activity of PPO was measured at different pH values during incubations at 30 °C for 5 min in 20 mM diethylbarbituric acid−HCl buffer (Britton−Robinson buffer). The optimum pH of the enzyme was found to be 5.0 (Figure 4). This value was different from those of raspberry [8.0 and 5.5 (*17*)], *Allium* sp. [7.5 (*20*)], amasya apple [7.0 (*4*)], Stanley plum [6.0 (*19*)], kiwi [6.8 and 7.3 (*14*)], field bean seed [4.0 (*24*)], and Jerusalem artichoke [6.0 (*27*)] using catechol as substrate. On the other hand, the optimum pH of Chinese cabbage PPO was similar to that of grape (*12*). The stability of the enzyme was examined by incubating the protein preparation at different pH values at 4 °C for 1 day in 20 mM Britton−Robinson buffer. As shown in Figure 4, the enzyme retained >80% of its original activity at pH 6.0 but lost its activity below pH 4.0 or above pH 7.0. Yang et al. (*10*) reported that banana PPO was stable at pH 5−11 at 5 °C for 48 h. Moreover, Fujita et al. (*28*) reported cabbage PPO was stable at pH 6−11 at 5 °C for 20 h. On the other hand, Rivas and Whitaker (*16*) reported the instability of pear PPO at pH <3.5. Siddiq et al. (*19*) reported plum PPO was unstable at pH <4.5. These results indicate that PPO was stable under neutral pH.

**Effect of Temperature.** The activity of PPO was measured at different temperatures at pH 5.0 for 5 min. The enzyme showed the highest activity at 50 °C (Figure 5). This value was different from those of amasya apple [18 °C (*4*)], grape [25 °C (*12*)], and Stanley plums [20 °C (*19*)]. The enzyme was incubated at different temperatures for 60 min, and after cooling, the residual enzyme activity was measured. Consequently, it was found that the enzyme was stable at 20 °C but was unstable at temperatures >50 °C (Figure 5). It has been reported that *Allium* sp. PPO was stable at 40 °C for 30 min (*20*), Stanley plum PPO at 70 °C (*19*), banana PPO at 70 °C for 30 min (*10*) and at 55 °C for 30 min (*9*), and Jerusalem artichoke PPO at 60 °C for 30 min (*27*).

**Substrate Specificity.** The substrate specificity of the enzyme was examined by using six chemicals (catechol, tyrosine, pyrogallol, dopamine, L-DOPA, and chlorogenic acid) as substrates. The substrate with higher activity was catechol, followed by pyrogallol, dopamine, and chlorogenic acid (Table 2). Michaelis constants ($K_m$) and maximum reaction velocities ($V_{max}$) were determined using these substrates under various concentrations (0.01, 0.03, 0.06, 0.08, and 0.1 M) and in optimum conditions (pH, temperature, and ionic strength). The Lineweaver−Burk plot analysis of this enzyme preparation showed $K_m$ values of 682.5 mM for catechol, 15.4 mM for pyrogallol, and 62.0 mM for dopamine (Table 3). The $K_m$ value of this enzyme against chlorogenic acid could not be measured. Pyrogallol was the most suitable substrate for Chinese cabbage PPO. This value for catechol was different from that of tea leaf [12.52 mM (*30*)], herb [25 mM (*20*)], amasya apple [34 mM (*4*)], Stanley plum [20 mM (*19*)], peach [2 mM (*11*)], spinach [3.13 mM (*21*)], field bean seed [10.5 mM (*24*)], and Jerusalem artichoke [4.0 mM (*27*)]. Chinese cabbage PPO showed the lowest affinity for catechol among them. It has been reported that $K_m$ values for pyrogallol are follows: apple, 27.0 mM (*4*); peach, 0.2 mM (*11*); spinach, 15.7 mM (*21*); and tea leaf, 17.8 mM (*30*). On the other hand, $K_m$ values for chlorogenic acid were different from those of apple [0.122 mM (*5*)], pear [5.9−11.7 mM (*16*)], spinach [11.6 mM (*21*)], and Jerusalem artichoke [1.8 mM (*27*)]. Moreover, $V_{max}$ values were 67.6 OD/min for catechol, 14.1 OD/min for pyrogallol, and 14.9 OD/min for dopamine, respectively (Table 4).

**Table 2. Substrate Specificity of Chinese Cabbage PPO**

| substrate | rel act. (%) | substrate | rel act. (%) |
|---|---|---|---|
| catechol | 100 | tyrosine | 13 |
| L-DOPA | 0 | dopamine | 55 |
| pyrogallol | 61 | chlorogenic acid | 15 |

**Table 3. $K_m$ and $V_{max}$ Values for Each Substrate**

| substrate | $K_m$ (mM) | $V_{max}$ (OD/min) |
|---|---|---|
| catechol | 682.5 | 67.6 |
| pyrogallol | 15.4 | 14.1 |
| dopamine | 62.0 | 14.9 |

**Inhibition Test of Browning.** Enzymatic browning of vegetables may be delayed or eliminated by removing the reactants such as oxygen and phenolic compounds or by using PPO inhibitors. Complete elimination of oxygen from vegetables during processing is difficult because oxygen is ubiquitous. Four compounds [ascorbic acid, L-cysteine, 2-mercaptoethanol (2-ME), and glutathione] were tested and percent inhibition values are shown in Table 4. The most effective inhibitor was

**Table 4. Inhibition of Browning by Reducing Reagents**

| inhibitor | concn (M) | % inhibition |
|---|---|---|
| ascorbic acid | $1.0 \times 10^{-4}$ | 49 |
| | $1.0 \times 10^{-3}$ | 51 |
| | $8.0 \times 10^{-3}$ | 75 |
| L-cysteine | $1.0 \times 10^{-4}$ | 41 |
| | $1.0 \times 10^{-3}$ | 43 |
| | $8.0 \times 10^{-3}$ | 44 |
| 2-mercaptoethanol | $1.0 \times 10^{-4}$ | 41 |
| | $1.0 \times 10^{-3}$ | 43 |
| | $8.0 \times 10^{-3}$ | 98 |
| glutathione | $1.0 \times 10^{-4}$ | 30 |
| | $1.0 \times 10^{-3}$ | 42 |
| | $8.0 \times 10^{-3}$ | 67 |

2-ME, followed by ascorbic acid, glutathione, and L-cysteine, in that order.

In conclusion, three PPO isozymes were detected by native PAGE in the crude extract of Chinese cabbage. The purified enzyme retained ~30% of its activity even at 50 °C. 2-ME effectively inhibited the browning. Because ascorbic acid is a naturally occurring water-soluble vitamin and nontoxic, it may be of use in preventing enzymatic browning of Chinese cabbage products.

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