## フキ（Petasites japonicus）のクロロゲン酸酸化酵素の精製と性質

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<th>項目</th>
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Purification and Characterization of Polyphenol Oxidase from Japanese Butterbur (Petasites japonicus)

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Polyphenol oxidase (o-diphenol: oxygen oxidoreductase, EC 1.10.3.1, PPO) in Japanese butterbur was purified about ~151-fold with a recovery rate of 1.13% by acetone fractionation, ion exchange chromatography, hydrophobic chromatography, and gel filtration. The purified enzyme appeared as a single band on PAGE and SDS-PAGE. The molecular weight of the enzyme was estimated to be about 26,000 and 25,000 by gel filtration and SDS-PAGE, respectively. The purified enzyme quickly oxidized chlorogenic acid and (-)-epicatechin. The Km values of the enzyme were 0.14 mM for chlorogenic acid (pH 5.0, 30°C) and 0.7 mM for (-)-epicatechin (pH 8.0, 30°C). The optimum pHs were 5.0 and 8.0 for chlorogenic acid oxidizing (ChO) and (-)-epicatechin oxidizing (EpO) activities, respectively. In the pH range from 4.0 to 9.0, both ChO and EpO activities were stable at 4°C for 22 h. The optimum temperature of both activities was found at 30°C. Both activities were 50% inactivated after heat treatment at 60°C for 10 min. Both activities were strongly inhibited by L-ascorbic acid and L-cysteine at 5 mM

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Key words: polyphenol oxidase, Japanese butterbur (Petasites japonicus), chlorogenic acid oxidase, (-)-epicatechin oxidase, purification and characterization

Enzymatic browning in raw fruits and vegetables is important in their preservation and processing, and is generally considered to be an undesirable reaction because it leads to unpleasant appearance and the concomitant development of off-flavors. Polyphenol oxidase (o-diphenol: oxygen oxidoreductase, EC 1.10.3.1, PPO) is found in most higher plants and is responsible for enzymatic browning. It catalyzes the oxidation of polyphenols in plants to o-quinones, which react with themselves or other phenolics to form a brown pigment. To prevent the browning that results in decreased marketability of the agricultural products, this enzyme has been characterized in many fruits and vegetables. Differences in the substrate specificity and some properties of PPO from various fruits and vegetables.

Japanese butterbur (Petasites japonicus) is one of the most common vegetables in Japan, and a brown scum caused by PPO always forms during its processing and/or cooking. However, details of the purification and properties of Japanese butterbur PPO have not hitherto been published. To determine the properties of PPO, pyrocatechol and 4-methylcatechol are generally used as substrate; however, chlorogenic acid (5-caffeoyl quinic acid) seems to be the normal substrate as it is widely distributed in plant tissues and is strongly oxidized by plant PPO. In Japanese butterbur, the presence of a few chlorogenic acid analogues has been reported, and the changes in absorption and difference spectra during the oxidation of Japanese butterbur extracts as well as...
of edible burdock extracts have indicated that the browning is caused by chlorogenic acid oxidation. Therefore, the enzymatic browning of Japanese butterbur seems to be mainly due to the oxidation of chlorogenic acid analogues by endogeneous PPO. In our laboratory, it has been clarified that the purified PPOs of composite vegetables, such as garland chrysanthemum, leaf lettuce and edible burdock, oxidize chlorogenic acid and (-)-epicatechin; however, the optimum pH of the enzyme differs depending on the type of substrate used. It is interesting that the same difference in optimum pH was observed in the purified PPO of Japanese butterbur. In this study, Japanese butterbur PPO was purified using chlorogenic acid as substrate, and some properties of the purified enzyme were determined.

**Materials and Methods**

1. **Materials**

Japanese butterbur was purchased at local markets in Saga Prefecture, Japan. DEAE-Cellulofine AL was purchased from Chisso, Tokyo, Japan. Butyl-Toyopearl 650 M, Phenyl-Toyopearl 650 M and Toyopearl HW 55-superfine (HW 55-S) were purchased from Tosco Co., Tokyo, Japan. The other reagents used were purchased from Wako Pure Chemical Co., Osaka, Japan and Katayama Chemical Co., Osaka, Japan.

2. **Assay of enzyme activity**

(1) **Chlorogenic acid oxidase (ChO) activity**

The spectrophotometric method developed by Tono et al. was employed to measure ChO activity. The mixture to be tested consisted of 0.5 mL of 0.4 mM aqueous solution of chlorogenic acid, 1 mL of 0.1 M citrate/0.2 M potassium phosphate buffer (McIlvaine buffer, pH 5.0) and 0.5 mL of the enzyme solution; it was incubated at 30°C for 5 min. After incubation, the reaction was stopped by adding 3 mL of 2% metaphosphoric acid solution. For the control, 0.5 mL of the enzyme solution was added to a mixture of 0.5 mL of 0.4 mM aqueous solution of chlorogenic acid, 1 mL of McIlvaine buffer (pH 5.0), and 3 mL of 2% metaphosphoric acid solution. The difference in absorbance at 325nm ($\Delta A_{325}$) between the control and test solutions was measured using a Shimadzu MPS-2000 spectrophotometer. One unit of the enzyme activity was defined as $\Delta A_{325}$ of 0.1 per minute and per milliliter of enzyme solution (1 cm light path).

(2) **PPO activity**

PPO activity was measured by the colorimetric method. The reaction mixture containing 0.5 mL of 10 mM aqueous solution of various polyphenols (see Table 2), 4 mL of McIlvaine buffer (pH 5.0), and 0.5 mL of enzyme solution. After 5 min of incubating the mixture at 30°C, the increase in absorbance at 420 nm ($\Delta A_{420}$) was measured using a Shimadzu MPS-2000 spectrophotometer. One unit of the enzyme activity was defined as $\Delta A_{420}$ of 0.1 per minute and per milliliter of enzyme solution (1 cm light path).

3. **Protein determination**

Protein was determined by the method of Hartree using bovine serum albumin (fraction V, Katayama Chemical Co., Osaka, Japan) as standard. In the chromatography, protein was determined by measuring the absorbance at 280 nm.

4. **Enzyme purification**

All steps of purification were carried out at 4°C. The edible part (leafstalk) of Japanese butterbur (3 kg) was homogenized in 1,500 mL of 0.1 M potassium phosphate/0.1 M sodium phosphate buffer (0.1 M phosphate buffer, PB, pH 7.0) containing 2% NaCl 1% L-ascorbic acid, and 1% polyvinyl polypyrrolidone. After filtering the homogenate through cotton cloth, the filtrate was centrifuged at 10,300 x g for 20 min, and acetone was added to the supernatant. The protein precipitate obtained from the 80% ammonium sulfate concentration and applied to a DEAE-Cellulofine AL column (4 x 14.5 cm) equilibrated with 0.01 M PB (pH 7.0), and eluted with a linear gradient of sodium chloride (0 to 1.0 M NaCl) in 0.01 M PB, pH 7.0. Enzyme activity was eluted with the buffer solution containing 0.5 to 0.8 M NaCl. The enzyme fractions were collected, brought to a 1 M ammonium sulfate concentration and applied to a Butyl-Toyopearl column (1.6 x 11 cm) equilibrated with 0.01 M PB (pH 7.0) containing 1 M ammonium sulfate and eluted with a linear gradient of ammonium sulfate (1 to 0 M in 0.01 M PB, pH 7.0). The enzyme was eluted with the buffer solution containing 0.6 to 0 M ammonium sulfate. The enzyme active fractions were pooled and dialyzed against 0.01 M PB (pH 7.0) with four or more changes of the dialyzed medium. The dialyzed
enzyme solution was brought to a 1.5 M ammonium sulfate concentration and applied to a Phenyl-Toyopearl column (1.6 x 11 cm) equilibrated with 0.01 M PB (pH 7.0) containing 1.5 M ammonium sulfate and eluted with a linear gradient of ammonium sulfate (1.5 to 0 M in 0.01 M PB, pH 7.0). The enzyme was eluted with the buffer solution containing 0.8 to 0.5 M ammonium sulfate. The enzyme active fractions were pooled and dialyzed against 0.01 M PB (pH 7.0) with four or more changes of the dialyzed medium. The dialyzed enzyme solution was applied to a Toyopearl HW 55-S (1.6 x 89 cm) column equilibrated with 0.1 M PB (pH 7.0) and eluted with the same buffer. The PPO active fractions were pooled and used for enzyme characterization.

5. Polyacrylamide gel electrophoresis (native PAGE)

The purified enzyme was electrophoresed by the method of DAVIS, using 7.5% polyacrylamide gel at pH 9.0.

6. Molecular weight determination

The molecular weight of the purified enzyme was estimated by gel filtration and SDS-PAGE. Gel filtration was conducted using a Toyopearl HW 55-S column (1.6 x 89 cm), which was equilibrated and eluted with 0.1 M PB (pH 7.0) by the method of ANDREWS. Chymotrypsinogen A (MW 25,000), egg albumin (MW 45,000), bovine serum albumin (MW 65,000), and γ-globulin (MW 125,000) were used as marker proteins at a flow rate of 20 ml/h. SDS-PAGE was carried out by the method of LAEMMLI, using an SDS marker protein kit (Oriental Yeast Co., Tokyo, Japan) as standard.

7. Assay of enzyme properties

(1) Optimum pH The effects of pH on ChO and EpO activities were determined at 30°C in McIlvaine (pH 3.0 to 8.0) and Atkins & Pantin buffers.

(2) pH stability The enzyme was preincubated in McIlvaine (pH 3.0 to 8.0) and Atkins & Pantin buffers at 4°C for 22 h. Residual ChO and EpO activities were determined under standard conditions (ChO: pH 5.0, 30°C; EpO: pH 8.0, 30°C).

(3) Optimum temperature ChO and EpO activities were determined at various temperatures (20 to 60°C) in McIlvaine buffer (ChO: pH 5.0; EpO: pH 8.0).

(4) Thermal stability The enzyme solution was heated at various temperatures between 30 to 80°C for 10 min and residual ChO and EpO activities were determined under standard conditions (ChO: pH 5.0, 30°C; EpO: pH 8.0, 30°C).

(5) Effect of various compounds ChO and EpO activities were measured in the presence (final concentration, 5 mM or 10 mM) and absence of various compounds under standard conditions (ChO: pH 5.0, 30°C; EpO: pH 8.0, 30°C).

Results and Discussion

1. Enzyme purification

Enzyme was purified from homogenates of Japanese butterbur by acetone fractionation, and DEAE-Cellulofine AL, Butyl-Toyopearl 650 M, Phenyl-

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**Table 1** Purification of PPO from Japanese butterbur

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total activity (unit)</th>
<th>Total protein (mg)</th>
<th>Specific activity (unit/mg protein)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80% acetone sulfate</td>
<td>250</td>
<td>15,900</td>
<td>2,288</td>
<td>7</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Cellulofine AL</td>
<td>563</td>
<td>9,188</td>
<td>37</td>
<td>248</td>
<td>16</td>
<td>35</td>
</tr>
<tr>
<td>Butyl-Toyopearl 650 M</td>
<td>563</td>
<td>2,432</td>
<td>4.6</td>
<td>528</td>
<td>75</td>
<td>15</td>
</tr>
<tr>
<td>Phenyl-Toyopearl 650 M</td>
<td>56</td>
<td>2,360</td>
<td>3.7</td>
<td>638</td>
<td>91</td>
<td>14.8</td>
</tr>
<tr>
<td>Toyopearl HW 55-S</td>
<td>700</td>
<td>1,797</td>
<td>1.7</td>
<td>1,057</td>
<td>151</td>
<td>11.3</td>
</tr>
</tbody>
</table>
Toyopearl 650 M and Toyopearl HW 55-S chromatographies. Fig. 1 shows the typical elution pattern of the enzyme activity on the final Toyopearl HW 55-S column. Enzyme activity showed one peak and the peak fractions from several columns were pooled as the purified PPO.

A summary of the typical purification of the enzyme is given in Table 1. Finally, the enzyme was purified ~151-fold with a recovery rate of 11.3%, relative to that of the crude enzyme.

2. Characterization of Japanese butterbur PPO

The purified enzyme produced a single band on native PAGE and SDS-PAGE (Fig. 2). These results suggest that Japanese butterbur PPO was purified to a homogeneous state. The molecular weight of the enzyme was estimated to be about 26,000 and 25,000 by gel filtration and SDS-PAGE, respectively (Fig. 3). These results indicate that the purified PPO is a monomer protein. The molecular weight is smaller than those of the PPOs of composite vegetables such as head lettuce (MW 56,000)\(^9\), garland chrysanthemum (MW 45,000)\(^19\), leaf lettuce (MW 46,000)\(^20\), and edible burdock (MW 40,000)\(^21\), all of which mainly oxidize chlorogenic acid. The molecular weight is also smaller than those of the PPOs of apples (MW 65,000)\(^22\), banana pulp (MW 41,000)\(^23\), and Japanese pears (MW 56,000)\(^24\), but is almost the same as that of another edible burdock PPO (MW 25,000)\(^25\) which mainly oxidize pyrogalol and phloroglucinol.

As shown in Table 2, the purified Japanese butterbur PPO quickly oxidized not only chlorogenic acid but also (−)-epicatechin. The enzyme had a low activity toward other o-diphenols such as pyrocatechol, catechin and caffeeic acid, but had no activity toward dopamine or DL-dopa. A similar substrate specificity was observed in the PPOs of composite vegetables such as head lettuce\(^6\), garland chrysanthemum\(^16\), leaf lettuce\(^16\) and edible burdock\(^17\), and of Japanese pears\(^9\). The enzyme also had no activity toward trihydroxybenzenes such as pyrogallol, gallic acid, and phloroglucinol, or m-diphenols such as resorcinol. The \(K_m\) values

\[\text{(A)}\]
\[
\begin{array}{c}
\text{M.W.} \\
\text{P.M} \\
\text{E}
\end{array}
\]

\[\text{(B)}\]
\[
\begin{array}{c}
\text{Origin} \\
\text{Front}
\end{array}
\]

![Fig. 2 SDS-PAGE (A) and native PAGE (B) of purified enzyme](image)

\(\text{M.W.: molecular weight, P.M.: protein marker,} \)

\(\Rightarrow: \text{purified enzyme.}\)

![Fig. 3 Estimation of molecular weight of the enzyme by gel filtration on Toyopearl HW 55-S (A) and SDS-PAGE (B)](image)

\(\text{(A): M.W.: molecular weight, Vo: void volume of the column, Ve: elution volume of the substance.} \)

\(\text{1: } \gamma \text{-globulin (160,000), 2: bovine serum albumin (65,000), 3: egg albumin (45,000), 4: chymotrypsinogen A (25,000), E: purified enzyme.} \)

\(\text{(B): 1: Cytochrome c hexamer (M.W.: 74,400), 2: Cytochrome c tetramer (49,000), 3: Cytochrome c trimer (37,200), 4: Cytochrome c dimmer (24,800), E: purified enzyme.} \)
Table 2 Substrate specificities of enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
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<tr>
<td>Chlorogenic acid</td>
<td>100</td>
</tr>
<tr>
<td>(−)-Epicatechin</td>
<td>94</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>42</td>
</tr>
<tr>
<td>Catechol</td>
<td>39</td>
</tr>
<tr>
<td>Catchin</td>
<td>37</td>
</tr>
<tr>
<td>Dopamin</td>
<td>9</td>
</tr>
<tr>
<td>DL-dopa</td>
<td>2</td>
</tr>
<tr>
<td>Resorcinolcinol</td>
<td>0</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>0</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>0</td>
</tr>
</tbody>
</table>

(Michaelis constant) of the enzyme, measured using chlorogenic acid (pH 5.0, 30°C) and (−)-epicatechin (pH 8.0, 30°C) as substrates, were 0.14 and 0.7 mM, respectively (Fig. 4). These values were lower than those of leaf lettuce PPO1 and edible burdock PPO2.

Several PPOs show different optimum pHs for different substrates: for example, the optimum pHs of garland chrysanthemum PPO for chlorogenic acid and epicatechin were 4.0 and 8.0, respectively. The optimum pHs of sweet pepper PPO for chlorogenic acid and pyrocatechol oxidation were 4.0 and 7.0, respectively. Fig. 5 (A) shows the effects of pH on the ChO and EpO activities of the purified Japanese butterbur PPO. The optimum pHs for ChO and EpO were 5.0 and 8.0, respectively. These optimum pH differences were also observed.

Fig. 4 Lineweaver-Burk plots of chlorogenic acid (A) and (−)-epicatechin (B) oxidation by the enzyme.

V: Rate of the enzyme reaction, S: substrate concentration.

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

○: ChO, chlorogenic acid oxidase activity; ●: EpO, (−)-epicatechin oxidase activity.

(A) PPO activity was measured at 30°C in McIlvaine (pH 3.0 to 8.0) and Atkins & Pantin (pH 9) buffers.

(B) PPO was preincubated in McIlvaine (pH 3.0 to 8.0) or Atkins & Pantin (pH 9) buffers at 4°C for 22 h. Residual ChO and EpO activities were measured under standard conditions (ChO: pH 5.0, 30°C; EpO: pH 8.0, 30°C).
in composite vegetables such as head lettuce\(^a\), leaf lettuce\(^b\) and edible burdock\(^c\), and eggplants\(^d\), On the other hand, the purified Japanese pear PPO\(^i\) had an optimum pH of 4.2 for the two substrates. The acidic optimum pHs for chlorogenic acid have also been reported for PPOs of eggplants\(^e\), apples\(^f\) and leaf lettuce\(^g\), which were found in the pH range of 4.0 to 5.0, for chlorogenic acid oxidation. In comparison with this, the optimum pHs of the PPOs of banana pulp\(^h\), banana peel\(^i\), and guava\(^j\) were found to be near neutrality using \((-\)\)-epicatechin, pyrocatechol, 4-methylecatechol, and dopamine as substrates. Therefore, the ChOs and EpOs in these plants were assumed to be acidic PPOs. Fig. 5 (B) shows the pH stabilities of the ChO and EpO activities of the purified PPO. Both activities were stable in the pH range of 4 to 9; \(-70\%\) of enzyme activity remained after incubation in solutions of various pHs from 3 to 9 for 22 h. The pH stability of the purified enzyme was different from that of garland chrysanthemum PPO\(^j\), the purified enzyme being stable in the pH range from 5 to 11. The pH stability of the purified enzyme was also slightly different from that of edible burdock PPO\(^j\), the enzyme being stable in the pH range from 5 to 7.

As shown in Fig. 6 (A), the purified enzymes showed a single optimum temperature with a maximum activity at 30°C, which is similar to the optimum temperatures of the PPOs of head lettuce\(^g\) and leaf lettuce\(^i\), but is slightly different from that of edible burdock PPO\(^j\) (20°C). ChO and EpO had slightly different thermal stabilities (Fig. 6 (B)). After heat treatment at 60°C, 70% of the EpO activity, but only about 50% of the ChO activity remained. The thermal stabilities of both ChO and EpO activities were relatively approximated, as observed in head lettuce\(^g\), garland chrysanthemum\(^j\) and leaf lettuce\(^i\).

The effects of various compounds on the purified enzyme activity are listed in Table 3. The ChO and EpO activities were markedly inhibited by sodium diethylidithiocarbamate, KCN, and NaF at 5 and 10 mM. The presence of metal ions (Zn\(^{2+}\), Cu\(^{2+}\), Ba\(^{2+}\), and Mn\(^{2+}\)) at 10 mM reduced ChO activity by 70%. The complete inhibition of ChO and EpO activities was induced by L-ascorbic acid and L-cysteine at 5 and 10 mM, respectively. Similar effects of these compounds were found for the PPOs of Japanese pears\(^j\), head lettuce\(^g\), garland chrysanthemum\(^j\), leaf lettuce\(^i\) and edible burdock\(^j\). Sodium chloride also markedly inhibited the ChO and EpO activities.

Considering the above-mentioned different effects of pH, temperature and various compounds on the ChO and EpO activities of the purified enzymes of composite vegetables and Japanese pear PPO, it appears that these enzymes may have separate sites for the two activities.

**Conclusion**

The PPO of Japanese butterbur was purified approximately 151-fold with a recovery rate of 11.3% using chlorogenic acid as a substrate. The molecular weight of the purified PPO (MW
Table 3 Effects of various compounds on the enzyme activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>ChO⁺ Relative activity (%)</th>
<th>(−) -EpO⁺ Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sodium diethylthiocarbamate</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>KCN</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>81</td>
<td>62</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>83</td>
<td>26</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>67</td>
<td>19</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>78</td>
<td>28</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>61</td>
<td>25</td>
</tr>
<tr>
<td>NaF</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>NaCl</td>
<td>93</td>
<td>93</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ ChO⁺: chlorogenic acid oxidase. The enzyme activity was determined at pH 5 using Mcllvaine buffer.
² EpO⁺: (-) -epicatechin oxidase. The enzyme activity was determined at pH 8 using Mcllvaine buffer.
³ Final concentration of the compound.
⁴ Not determined.

25,000) is smaller than those of the PPOs of composite vegetables such as head lettuce (MW 56,000)¹, garland chrysanthemum (MW 45,000)¹, leaf lettuce (MW 46,000)¹, and edible burdock (MW 40,000)¹: however, the substrate specificity of the purified PPO, and the effects of pH, temperature, and various compounds on the purified enzyme activity were similar to those on the enzymes of the composite vegetables. These results suggest that the browning of Japanese butterbur is caused by the oxidation of chlorogenic acid and its analogues, and that L-ascorbic acid and L-cysteine are effective inhibitors of such enzymatic browning.

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フキ（*Petasites japonicus*）のクロロゲン酸酸化酵素の精製と性質

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