Purification and Characterization of Chlorogenic Acid Oxidase from Edible Burdock (Arctium lappa L.)

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The spectral profiles of edible burdock extract during browning reaction suggested that the oxidation of chlorogenic acid and its analogues mainly causes enzymatic browning in edible burdock. Polyphenol oxidase (PPO) was purified ~16.6-fold with a recovery rate of 21% using chlorogenic acid as substrate. The purified enzyme appeared as a single band on PAGE and SDS-PAGE. The molecular weight of the enzyme was estimated to be about 41,000 and 40,000 by gel filtration and SDS-PAGE, respectively. The purified enzyme quickly oxidized chlorogenic acid and (−)-epicatechin. The Km values of the enzyme were 0.4 mM for chlorogenic acid (pH 5.0, 20°C) and 2.7 mM for (−)-epicatechin (pH 8.0, 20°C). The optimum pHs were 5.0 for chlorogenic acid oxidase (ChO) and 8.0 for (−)-epicatechin oxidase (EpO). In the pH range from 5 to 8, both ChO and EpO activities were quite stable at 4°C for 22 h. The optimum temperature of both activities was 20°C. Both activities were 50% inactivated after a heat treatment at 45°C for 30 min. Both activities were strongly inhibited by L-ascorbic acid and L-cysteine at 5 mM.

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(HW 55-S) were purchased from Toso Co., Tokyo, Japan. The other reagents used were purchased from Wako Pure Chemical Co., Osaka, Japan, and Katayama Chemical Co., Osaka, Japan.

2. Measurement of absorption and difference spectra of brown solution of edible burdock extract

(1) Preparation of edible burdock extract
Fifty grams of sliced (2 mm thick) fresh edible burdock was heated using a microwave oven for 3 min to inactivate PPO, and homogenized in 200 ml of ethanol. The resulting slurry was boiled under reflux for 3 h, and filtered through Toyo No. 2 filter paper. The residue on the paper was washed three times with 100 ml of ethanol. All fractions of the filtrate were combined, concentrated in vacuo to about 20 ml, and diluted with distilled water to 100 ml.

(2) Measurement of spectra
Crude PPO solution (0.5 ml) (see enzyme preparation section) was added to a mixture of 0.5 ml of the edible burdock extract, 4 ml of 0.1 M citrate-0.2 M sodium phosphate buffer (McIlvaine buffer, pH 5) and 5 ml of 2% metaphosphoric acid (HPO₃). Because PPO was inactivated in the mixture by HPO₃, no browning of the mixture was observed. One milliliter of the resulting mixture was diluted with 4 ml of 2% HPO₃ (control solution). Crude PPO solution (0.5 ml) was added to a mixture of 0.5 ml of edible burdock extract and 4 ml of McIlvaine buffer (pH 5). After incubation for 5 min at 30°C, 5 ml of 2% HPO₃ was added to the mixture to stop the browning reaction. One ml of the resulting mixture was diluted with 4 ml of 2% HPO₃ (test solution). The absorption spectra and difference spectra of control and test solutions were measured using a Shimadzu MPS-2000 spectrophotometer.

The absorption spectra and difference spectra of the chlorogenic acid solution during enzymatic browning reactions were determined by the same method using 0.5 ml of 0.4 mM chlorogenic acid solution instead of 0.5 ml of edible burdock extract.

3. 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 chlorogenic acid, 1 ml of McIlvaine buffer (pH 5) and 0.5 ml of the enzyme solution, and was incubated at 30°C for 5 min. After incubation, the reaction was stopped by the addition of 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 chlorogenic acid solution, 1 ml of McIlvaine buffer (pH 5), and 3 ml of 2% metaphosphoric acid solution. The difference in absorbance at 325 nm (ΔA₃25) between the control and test solutions was measured using a Shimadzu MPS-2000 spectrophotometer. One unit of the enzyme activity was expressed as ΔA₃25 of 0.1 per minute per milliliter of the enzyme solution (1 cm light path).

(2) PPO activity
PPO activity was measured by the colorimetric method for 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), and 0.5 ml of enzyme solution. After 5 min of incubation of the mixture at 30°C, the increase in absorbance at 420 nm (ΔA₄20) was measured using a Shimadzu MPS-2000 spectrophotometer. One unit of the enzyme activity was defined as ΔA₄20 of 0.1 per minute and per milliliter of enzyme solution (1 cm light path).

4. Assay of enzymes properties

(1) Optimum pH
The effects of pH on the ChO and EpO activities of the PPO were determined at 20°C in McIlvaine (pHs 3.0 to 8.0) and Atkins & Pantin (pH 9.0) buffers.

(2) pH stability
The enzyme was preincubated in McIlvaine (pH 3.0 to 8.0) and Atkins & Pantin (pH 9.0) buffers at 4°C for 22 h. Residual ChO and EpO activities were measured under standard conditions (ChO: pH 5.0, 20°C; EpO: pH 8.0, 20°C).

(3) Optimum temperature
The enzyme was assayed at various temperatures (20 ~ 60°C) in McIlvaine buffer (ChO: pH 5.0; EpO: pH 8.0).

(4) Temperature stability
The enzyme solution was heated at various temperatures between 30 and 80°C for 30 min at pH 7.0. Residual ChO and EpO activities were determined under standard conditions (ChO: pH 5.0, 20°C; EpO: pH 8.0, 20°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, 20°C; EpO: pH 8.0, 20°C).
5. Protein determination

Protein was determined by the method of HARTREE\textsuperscript{13} 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.

6. Enzyme purification

All steps of purification were carried out at 4 °C. The edible part of edible burdock (fresh weight, 1.5 kg) was homogenized with 1,500 ml of 0.1 M potassium phosphate–0.1 M sodium phosphate buffer (0.1 M phosphate buffer, PB, pH 7) containing 2% NaCl, 1% l-ascorbic acid, and 1% polyvinyl polypyrrolidone. After filtration of the homogenate through cotton cloth, the filtrate was centrifuged at 8,000 g for 20 min, and acetone was added to the supernatant. The protein precipitate obtained from the 20–80% acetone fraction was collected by centrifugation at 8,000 g for 20 min, dissolved in a small volume of 0.01 M PB (pH 7), and then dialyzed against the same buffer for 36 h with four or more changes of the dialysis medium. The dialyzed solution (crude enzyme) was applied to a DEAE-Cellulofine AL column (4 × 14.5 cm) equilibrated with 0.01 M PB (pH 7), and eluted with a linear gradient of sodium chloride (0 to 1.0 M NaCl in 0.01 M PB, pH 7). The 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 × 11 cm) equilibrated with 0.01 M PB (pH 7) 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). 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) with four or more changes of the dialysis medium. The dialyzed enzyme solution was applied to a Toyopearl HW 55-S (1.6 × 89 cm) column equilibrated with 0.1 M PB (pH 7) and eluted with the same buffer. The PPO active fractions were pooled and used for enzyme characterization.

7. Polyacrylamide gel electrophoresis (PAGE)

An electrophoresis of the purified enzyme was carried out by the method of DAVIS\textsuperscript{16}, using 7.5% polyacrylamide gel at pH 9.

8. 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 × 89 cm), which was equilibrated and eluted with 0.1 M PB (pH 7), by the method of ANDREWS\textsuperscript{15}. 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 described by LAEMMLI\textsuperscript{16}, using an SDS marker protein kit (Oriental Yeast Co., Tokyo, Japan) as standard.

Results and Discussion

1. Changes of absorption and difference spectra during browning reaction

A marked browning of the reaction mixture of the edible burdock extract by its PPO was observed. During the browning reaction, characteristic changes in the absorption and difference spectra of the reaction mixture were also observed. As shown in Fig. 1-1, the absorbance of the reaction mixture of the edible burdock extract decreased in the range from 260 nm to 360 nm during the browning reaction. In the difference spectra, a negative peak was found at 325 nm and positive

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Changes in Absorption and Difference Spectra of Edible Burdock Extract and Chlorogenic Acid Solution During Browning Reaction by Edible Burdock Polyphenol Oxidase}
\end{figure}

Fig. 1–I Edible burdock extract : (A) absorption spectra : a : 0 min, b : 5 min. (B) difference spectra.

Fig. 1–II Chlorogenic acid solution : (A) absorption spectra : a : 0 min, b : 5 min. (B) difference spectra.
peaks were found at 260 nm and 360 nm (Fig. 1-I B). FUJITA and TONO reported the spectral profiles of brown solutions of various polyphenolic compounds during an oxidative reaction caused by Satsuma mandarin PPO. The spectral profiles of edible burdock extract during the browning reaction were similar to those of chlorogenic acid (Fig. 1-II) solution, and different from those of other polyphenols such as pyrocatechol, pyrogallol, and DL-dopa. NAKABAYASHI detected chlorogenic acid analogues in large quantities in edible burdock, which were quickly oxidized by its crude PPO. These results suggest that the oxidation of chlorogenic acid and its analogues mainly cause enzymatic browning in edible burdock. Similar spectral profiles were reported for Satsuma mandarin, head lettuce, and Japanese pear during the enzymatic oxidation of their extract by their PPO. Therefore, edible burdock PPO were purified and characterized using chlorogenic acid as substrate in this experiment.

2. Enzyme purification

Enzyme was purified from the homogenates of edible burdock by acetone fractionation, and DEAE-Cellulofine, Butyl-Toyopearl 650 M, and Toyopearl HW 55-S gel filtrations. Typical results of the stepwise purification of the enzyme are given in Table 1. Finally, the enzyme was purified ~16.6-fold with a recovery rate of 21%, as compared with the crude enzyme.

3. Some properties of edible burdock PPO

The purified enzyme produced a single band on PAGE and SDS-PAGE (Fig. 2). These results suggest that edible burdock PPO was purified to a homogeneous state. The molecular weight of the enzyme was estimated to be about 40,000 and 41,000 by gel filtration and SDS-PAGE, respectively (Table 3). These results indicate that the purified PPO is a monomer protein. The molecular weight of the purified PPO was different from that of another edible burdock PPO purified by MURAO et al., the molecular weight of which was estimated to be about 25,000 and 31,000 by gel filtration and SDS-PAGE, respectively. The molecular weight was smaller than those of the PPOs of head lettuce (MW 56,000) and garland chrysanthemum (MW 45,000), and apple (MW 65,000), all of which mainly oxidize chlorogenic acid. As shown in Table 2, our purified edible burdock PPO quickly oxidized not only chlorogenic acid but also (-)-epicatechin. The enzyme had a low activity toward

<table>
<thead>
<tr>
<th>Procedure step</th>
<th>Volume (µL)</th>
<th>Total activity (units)</th>
<th>Total protein (µg)</th>
<th>Specific activity (units/mg protein)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20~80% acetone sulfate</td>
<td>126</td>
<td>4,361</td>
<td>78</td>
<td>56</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Cellulofine AL</td>
<td>129</td>
<td>3,256</td>
<td>23</td>
<td>142</td>
<td>2.5</td>
<td>75</td>
</tr>
<tr>
<td>Butyl-Toyopearl 650 M</td>
<td>58</td>
<td>1,813</td>
<td>3</td>
<td>604</td>
<td>10.8</td>
<td>42</td>
</tr>
<tr>
<td>Toyopearl HW-55 S</td>
<td>232</td>
<td>930</td>
<td>1</td>
<td>930</td>
<td>16.6</td>
<td>21</td>
</tr>
</tbody>
</table>

![Fig. 2](image)

**Table 1** Purification of Ch0 from Edible Burdock

**Table 2** Substrate Specificities of Enzyme
other o-diphenols such as catechol and caffeic acid, but had no activity toward catechin, dopamine, or DL-dopa. A similar substrate specificity was observed in the PPO of Japanese pears\(^{10}\), head lettuce\(^{3}\), and garland chrysanthemum\(^{10}\). The enzyme also had no activity toward trihydroxybenzenes such as pyrogallol, gallic acid, and phloroglucinol, and m-diphenol such as resorcinol. The substrate specificity of our PPO was different from another edible burdock PPO\(^{11}\), which only oxidized trihydroxybenzenes such as pyrogallol, gallic acid, and phloroglucinol (so we call this enzyme trihydroxybenzene oxidase (TBO) in the text).

The \(K_m\) values (Michaelis constant) of the enzyme, measured using chlorogenic acid (pH 5.0, 20\(^\circ\)C) and \((-\)epicatechin (pH 8.0, 20\(^\circ\)C) as substrates, were 0.4 and 2.7 mM, respectively. These values were lower than those of head lettuce PPO\(^{6}\), and garland chrysanthemum PPO\(^{10}\).

Some properties of the enzymes are summarized in Table 3. Several PPOs show different optimum pHs for different substrates: for example, the optimum pHs of sweet pepper PPO for chlorogenic acid and procatechol were 4.0 and 7.0, respectively\(^{20}\). The optimum pHs of garland chrysanthemum PPO for ChO and EpO were 5.0 and 8.0, respectively\(^{10}\). The optimum pHs of purified edible burdock PPO were 5.0 and 8.0 for ChO and EpO, respectively. On the other hand the purified Japanese pear\(^{7}\) had a pH optimum of 4.2 for the two substrates. The acidic optimum pHs of the PPOs for chlorogenic acid have also been reported for PPOs of eggplant\(^{15}\) and apple\(^{15}\), which were found in the pH range of 4.0 to 5.0, for chlorogenic acid oxidation. In comparison with this, the optimum pH was found to be near neutrality for the PPOs of banana pulp\(^{7}\), banana peel\(^{15}\), and guava\(^{11}\), using \((-\)epicatechin, pyrocatechol, 4-methylcatechol, and dopamine as substrates. Therefore, ChOs in these plants assumed to be acidic PPOs. The optimum pH of the enzyme was also different from that of TBO, which was found to be 7\(^{11}\).

The activities of ChO and EpO were stable in the pH range of 5 to 7; \(~70\%\) 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 those of TBO, the purified enzyme being stable in the pH range from 7 to 9.

Murao et al.\(^{11}\) also reported that the purified TBO had an optimum temperature of 60\(^\circ\)C and was relatively stable at high temperatures: about 50\% of the activity remained after heat treatment at 55\(^\circ\)C for 30 min. In contrast to this, our PPO had an optimum temperature of 20\(^\circ\)C, and was more unstable than that of TBO at high temperatures: about 50\% of the activity remained after heat treatment at 45\(^\circ\)C for 30 min. Similar thermal stabilities have also been reported for the PPO of head lettuce\(^{3}\), however, ChO and EpO slightly differed in stability.

The effects of various compounds on the purified enzyme activity are listed in Table 4. The activities of ChO and EpO were markedly inhibited by sodium diethyldithiocarbamate, KCN, and NaF at 5 and 10 mM. Metal ions (Ba\(^{2+}\) and Mn\(^{2+}\)) strongly inhibited ChO activity at 5 mM. 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 PPOs of Japanese pear\(^{5}\), head lettuce\(^{9}\), banana pulp\(^{7}\), banana peel\(^{15}\), and garland chrysanthemum\(^{16}\). Sodium chloride also markedly inhibited the activities of ChO and EpO.

The results mentioned above suggest that the

<table>
<thead>
<tr>
<th>Table 3 Some Properties of Enzyme</th>
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<tr>
<td>Molecular weighta</td>
</tr>
<tr>
<td>General property</td>
</tr>
<tr>
<td>Optimum pH</td>
</tr>
<tr>
<td>pH stability</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
</tr>
<tr>
<td>Temperature stability (°C)</td>
</tr>
</tbody>
</table>

a Determined by SDS-PAGE^ and gel filtration^.
b ChO: chlorogenic acid oxidase activity. c EpO: \((-\)epicatechin oxidase activity.
d 70\% remaining. e 50\% remaining.
molecular weight and other properties of the purified PPO were quite different from those of the edible burdock PPO (trihydroxybenzene oxidase) purified by Murao et al.\textsuperscript{11) The results also suggest that the browning of edible burdock is caused by the oxidation of chlorogenic acid and its analogues, and that L-ascorbic acid and L-cysteine are effective inhibitors of the enzymatic browning in edible burdock.

References


Table 4 Effect of Various Compounds on Enzyme

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Relative activity (%)</th>
<th>ChO&lt;sup&gt;a&lt;/sup&gt;</th>
<th>(-)-EpO&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10 mM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5 mM&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sodium diethylthiocarbamate</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KCN</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>8</td>
<td>12</td>
<td>71</td>
</tr>
<tr>
<td>ZnSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>96</td>
<td>66</td>
<td>.&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CuSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>80</td>
<td>49</td>
<td>68</td>
</tr>
<tr>
<td>BaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>13</td>
<td>2</td>
<td>.&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>MnCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10</td>
<td>3</td>
<td>.&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaF</td>
<td>18</td>
<td>0</td>
<td>86</td>
</tr>
<tr>
<td>NaCl</td>
<td>34</td>
<td>23</td>
<td>60</td>
</tr>
<tr>
<td>L-Ascorbic acid</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

a ChO: chlorogenic acid oxidase activity. The enzyme activity was determined at pH 5.
b (-)-EpO: (-)-epicatechin oxidase activity. The enzyme activity was determined at pH 8.
c Final concentration of compound.
d Not determined.
Characterization of Edible Burdock ChO


15) ANDREWS, P.: The gel-filtration behavior of proteins related to their molecular weight over wide range, Biochem. J., 96, 595–605 (1965)


ゴボウ (Arctium lappa L.) のクロロゲン酸酸化酵素の精製とその性質

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ゴボウの抽出液の褐変反応中のスペクトルの特徴からクロロゲン酸をあらゆる同族体の酸化反応が酵素の褐変の主原因であることが示唆された。クロロゲン酸を基質としてゴボウのポリフェノール酸化酵素 (PPO) は16、6倍に精製され、その回収率は約21%であった。精製酵素のPAGEおよびSDS-PAGEにおいて、1本のバンドを示した。精製酵素の分子量はゲル通過法とSDS-PAGEによりそれぞれ41,000と40,000と算出された。精製酵素はクロロゲン酸およびエビカテキンを速やかに酸化した。酵素のクロロゲン酸 (pH 5.0, 20℃) とエビカテキン (pH 8.0, 20℃) に対するKm値は0.4と2.7 mMと算出された。クロロゲン酸酸化酵素 (ChO) 活性の最適pHは5.0, エビカテキン酸化酵素 (EpO) 活性の最適pHは8.0に認められた。本酵素を4℃で22時間処理した後、pHs 5.0～8.0範囲で安定であった。両活性の最適温度は20℃に認められた。45℃で30分加熱を行った結果、両活性の約50%が失活した。両活性は、5 mMのL-アスコルビン酸, L-システインによって強く抑制された。

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