Subcellular Location of Polyphenol Oxidase in Apples

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The location of polyphenol oxidase (PPO) in cells of apple fruit was examined by immunohistochemistry and subcellular fractionation. In mature apple fruits, where vacuoles occupy most of the cells, PPO was detected immunohistochemically near the cell walls with use of anti-apple PPO antibodies. In cells of immature fruits and tissue culture, PPO was detected in organelles other than the vacuoles, probably in plastids. The plastid fraction was purified by density gradient ultracentrifugation, and the activities of PPO and marker enzymes of plastids were the highest in the plastids. Most apple PPO was in plastids, as are other plant PPOs, and some of the protein was solubilized and proteolyzed during ripening and storage.

Key words: polyphenol oxidase; apple (Malus pumila); plastid; subcellular location; enzymatic browning

Polyphenol oxidase (EC 1.10.3.2, PPO) is found in various kinds of plants, and oxidizes polyphenols to their corresponding quinones. These quinones are polymerized with quinones or amines to form brown pigments. PPO is responsible for this enzymatic browning, often observed in fruit and vegetables such as apples, bananas, and lettuce at the table. The regulation of this browning is important for food processing. The substrate of the enzyme, polyphenols, are in the vacuoles, and the enzyme is in plastids or chloroplasts. Accordingly, the enzyme and its substrate do not meet and so intact cells do not turn brown. When cells are broken by being cut or bruised, the browning reaction starts.

PPO is generally in plastids or chloroplasts in intact cells. However, there is little experimental evidence showing the location of PPO in apple cells. Harel et al. suggested that apple PPOs are in plastids and mitochondria because of the results of cellular fractionation. We have isolated apple PPO as chlorogenic acid oxidase of mature fruits of apples. We next prepared antibodies against apple PPO and examined immunohistochemically the distribution of PPO at the tissue level in mature and immature fruits of apples. Here we report the subcellular location of PPO in apples, found by immunohistochemical assays and subcellular fractionation.

Materials and Methods

Materials. Immature apples (Malus pumila Mill. var. domestica) Schneid. cv. Fuji, 4.5 mo after flowering were obtained from the Fruit Tree Research Station, Morigaoka Branch (Morigaoka, Japan) in 1994. Mature apples (Fuji) were purchased in a local Tokyo market in 1995 and 1996. Apple cells (Fuji) in suspension culture were subcultured in our laboratory for about two years and used.

Immunohistochemistry. Sections (3 x 4 mm) of mature apples were fixed, dried, embedded, and sectioned as described in a previous paper. Sections (about 1 x 1 cm) of immature apples were fixed with a 2:1:10:7 solution of formalin, acetic acid, ethanol, and water (FAE). After sections were washed 3 times with phosphate-buffered saline (PBS) consisting of 0.85% NaCl, 0.02% KCl, 0.02% KH₂PO₄, and 0.2% Na₂HPO₄ 12H₂O (pH 7.2), the sections were treated with 0.5% Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo) in PBS for 30 min at 45°C with gentle shaking. Liberated cells were put onto glass slides and dried overnight.

Apple cells from a suspension culture were fixed with FAE solution for 2 h at room temperature. After cells were washed with PBS 3 times, they were dehydrated in a graded series of ethanol concentrations (2.5, 5, 10, 20, 40, 50, 60, 70, 80, 90, 95, 99, 99.5, and 100%). The cells were put onto glass slides in a drop of water and dried.

Apple cells or sections were treated with anti-apple PPO antibodies, stained with anti-mouse IgG conjugated with fluorescein isothiocyanate (Dakopatts, Denmark), and observed under a fluorescence microscope (Olympus BH-2, Tokyo) as already described.

Subcellular fractionation. An apple was sliced, cored, and homogenized with a juicer-blender (Tescan TM90, Tokyo) for 30 s in a 0.1 M Na/K phosphate buffer (pH 7.2) containing 0.4 M sucrose and 0.01 M ascorbic acid. The homogenate was filtered with 4 sheets of gauze. The filtrate (total homogenate) was centrifuged at 500 x g for 20 min. The precipitate that formed was washed with the extraction buffer, and designated the crude plastid fraction. The supernatant from the total homogenate was centrifuged at 39,000 x g for 30 min and the precipitate was designated the mitochondrion fraction. To the supernatant, ammonium sulfate was added to 80% saturation, and the precipitate that formed was recovered by centrifugation at 9000 x g for 10 min, and designated the soluble fraction. This crude separation was repeated for at least ten apples.

The crude plastid fraction was further purified by the method of Journet and Douce with some modifications. The crude plastid was suspended with medium B consisting of 0.3 M sucrose, 1 mM EDTA, 0.1% bovine serum albumin, and 0.01 M potassium phosphate buffer (pH 7.2), layered over 35% (v/v) Percoll in medium B, and centrifuged at 46,000 x g for 30 min in an ultracentrifuge (Beckman Optima L-80) with an angle rotor (Beckman Optima L-80, 70TI). The yellowish plastid fraction was taken and washed with medium B. The pellet was suspended in medium B, layered over 35% Percoll in medium B, and centrifuged at 29,000 x g for 2 h in a swing rotor (Beckman SW41TI). The yellowish band was taken, washed with medium B, and used as purified plastids.

Enzyme activity and protein assay. PPO activity was measured as described earlier. PPO was assayed at pH 4.0 and 7.0, because purified PPO from crude plastids had optimal pH 4.0 and mitochondrial PPO had optimal pH 7.0. Fumarase activity, a marker for mitochondria, was measured by the spectrophotometric method at 230 nm to detect fumaric acid. The reaction solution consisted of 1.6 ml of 0.3 M sucrose in 50 mM MOPS-NaOH (pH 7.3), 0.2 ml of enzyme solution, and 0.2 ml of 450 mM maleic acid. Activity causing an increase in absorbance of 0.1 per minute at 250 nm and 30°C was defined as 1 unit of fumarase activity. Catalase activity, a marker for microbodies, was measured by the spectrophotometric method at 240 nm to detect H₂O₂. The reaction solution con-
sisted of 1.8 ml of 0.3 m sucrose in 50 mM MOPS-NaOH (pH 7.0), 0.1 ml of enzyme solution, and 0.1 ml of 1% H₂O₂. Activity causing a decrease in absorbance of 0.1 per minute at 240 nm and 30°C was defined as 1 unit of catalase activity. Glucose-6-phosphate dehydrogenase activity, a marker for plastids, was measured by the spectrophotometric method at 340 nm to detect NADPH. The reaction solution consisted of 1.2 ml of 0.5 M sucrose in 83 mM Tricine-NaOH (pH 8.2), 0.2 ml of MgCl₂, 0.2 ml of 5 mM NADP, and 0.2 ml of enzyme solution. Activity causing an increase in absorbance of 0.1 per minute at 340 nm and 30°C was defined as 1 unit of glucose-6-phosphate dehydrogenase activity. Glycerolphosphate dehydrogenase activity, a marker for plastids, was measured by the spectrophotometric method at 340 nm to detect NADPH. The reaction solution consisted of 1.1 ml of 0.68 M sucrose in 90 mM Tris-HCl (pH 7.5), 0.2 ml of 100 mM MgSO₄, 0.2 ml of 65 mM cysteine, 0.2 ml of 13 mM glutathione, 0.2 ml of 2 mM NADPH, 0.2 ml of 13.75 mM ATP, 0.2 ml of enzyme solution, and 0.2 ml of 112.5 mM 3-phosphoglyceric acid. Activity causing an increase in absorbance of 0.1 per minute at 340 nm and 25°C was defined as 1 unit of glycerolphosphate dehydrogenase activity. Nitrite reductase activity, a marker for plastids, was measured by the method of Vega et al. The reaction solution consisted of 1.15 ml of 0.3 M sucrose in 50 mM Tricine-NaOH (pH 8.0), 0.2 ml of 15 mM NaNO₂, 0.15 ml of 19 mM methylviologen, 0.3 ml of 25 mg/ml sodium hydrosulphite in 0.29 M NaHCO₃ and 0.2 ml of enzyme solution. After incubation at 30°C for 10 min, a portion was sampled and mixed with diazo-coupling reagent; the loss of absorbance of the diazo dye compound was measured at 540 nm. Activity causing a decrease in 1.1 mmol per minute was defined as 1 unit of nitrite reductase activity.

Each measurement was done in duplicate. Coefficients of variation were less than 0.1. Each enzyme activity was determined in the absence or presence of detergent (0.025-0.5% Triton X-100), because detergent broke the structure of cellular organelles.

Protein content was assayed by the Lowry method with bovine serum albumin as the standard.

**Electrophoresis and Western blotting.** SDS-PAGE and Western blotting were done as described previously. The crude plastid fraction and soluble fraction were electrophoresed under completely denatured conditions and blotted onto nitrocellulose membranes. PPO on the membrane was detected by the mouse antibodies against apple PPO, anti-mouse IgG complexed with peroxidase, and a mixture of diaminobenzidine and H₂O₂.

**Results**

**Immunocytochemical findings.**

Figure 1 shows PPO in apple cells of a mature fruit. Fluorescence was detected only near the periphery of the cells, suggesting that cells were occupied by vacuoles. When the section was treated with nonimmune serum, no clear fluorescence was not seen. Figure 2A is a micrograph of apple cells of a suspension culture. Vacuoles occupied far less of the cytosol in cultured cells than in cells of mature fruit. Figure 2B shows PPO in apple cells of a suspension culture. PPO was seen in some organelles. Figure 3 shows PPO in apple cells of an immature fruit. In this stage, organelles other than vacuoles are seen (Fig. 3A), although vacuoles occupied most parts of cells. PPO was seen only in organelles other than vacuoles (Fig. 3B). When cells were treated with nonimmune serum, there was little fluorescence (Fig. 3C).

**Subcellular fractionation.**

In most apples we examined, the specific activities of PPO and glucose-6-phosphate dehydrogenase were the highest in the crude plastid fraction with Triton X-100 added (Table I). The mitochondrion fraction also contained much PPO, but the activities of PPO and glucose-6-phosphate dehydrogenase were raised less by addition of Triton X-100. The activities of fumarase and catalase also were high in the crude plastid fraction, and they were increased a little by Triton X-100. Fumarase and catalase activities were higher in the mitochondrion fraction, when Triton X-100 was present. The soluble fraction we obtained may have contained microsomes.

The PPO activity at pH 4.0 was higher than that at pH 7.0 in all fractions. Therefore, we further purified plastids in the crude plastid fraction by Percoll-density gradient ultracentrifugation done twice. The purified plastid fraction showed the highest PPO activity of all, and it also had the highest activities of glucose-6-phosphate dehydrogenase.

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*Fig. 1.* Immunocytochemical Staining of PPO in a Mature Apple. This section was stained by the indirect immunofluorescence method.

*Fig. 2.* Immunocytochemical Location of PPO in Cultured Apple Cells. A, not stained; B, stained by the indirect immunofluorescence method using anti-apple PPO antibody.
Table I. PPO Distribution in Crude Separation of Mature Apples

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Triton X-100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
</tr>
<tr>
<td>PPO, pH 4.0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td>PPO, pH 7.0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td>Fumarase</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>Catalase</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td>Gluconate-6-phosphate dehydrogenase</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td>Total protein (mg/100 g)</td>
<td>457</td>
</tr>
</tbody>
</table>

Means of five apples are shown. n.d., not determined.

Table II. Purification of Plastids in a Mature Apple and PPO Activity

<table>
<thead>
<tr>
<th>Enzyme (location)</th>
<th>Total homogenate</th>
<th>Crude plastid</th>
<th>Purified plastid</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPO (mitochondria)</td>
<td>n.d.</td>
<td>6.1</td>
<td>42.3</td>
</tr>
<tr>
<td>Fumarase (microsomes)</td>
<td>7.4</td>
<td>5.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Catalase (microsomes)</td>
<td>3.9</td>
<td>12.0</td>
<td>12.2</td>
</tr>
<tr>
<td>Gluconate-6-phosphate dehydrogenase (plastids)</td>
<td>0.5</td>
<td>1.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (plastids)</td>
<td>0.1</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Nitrite reductase (plastids)</td>
<td>—</td>
<td>—</td>
<td>0.2</td>
</tr>
<tr>
<td>Total protein (mg/100 g apple)</td>
<td>493</td>
<td>25</td>
<td>0.7</td>
</tr>
</tbody>
</table>

n.d., not determined; —, not detected; Triton X-100 (0.025–0.5%) was added for the enzyme assay.

Fig. 3. Immunocytochemical Location of PPO in an Immature Apple. A, not stained; B, stained by the indirect immunofluorescence method using anti-apple PPO antibodies; C, stained by indirect immunofluorescence method using nonimmune serum.

glyceraldehyde-3-phosphate dehydrogenase, and nitrite reductase (Table II). Except for the yellowish plastid fractions, obtained by ultracentrifugation, did not show PPO and gluconate-6-phosphate dehydrogenase activities. However, the recovery of plastids, estimated by the activities of the marker enzymes, during purification was low. Fumarase activity was lower in purified plastids than in crude plastids, and catalase activity was about the same in both fractions.

Fig. 4. Western Blotting Analysis of Apple PPO. A, soluble fraction; B, crude plastid fraction.
In some retail apples examined, PPO activity was the highest in the soluble fraction. Activities of PPO, fumarase, catalase, and gluconate-6-phosphate dehydrogenase were not increased by Triton X-100 added to the soluble fraction of these apples. The protein content of the soluble fraction was about twice higher than that in most apples we tested. In Western blotting of PPO, a band at about 65 kDa was detected in the crude plastid fraction and two bands at about 43 and 65 kDa were detected in the soluble fraction with high PPO activity (Fig. 4).

**Discussion**

Cellular fractionation studies done with differential or gradient centrifugation suggest that pellets enriched in chlorophyll or other plastid markers are enriched in PPO. For example, Henry et al.\(^{15}\) showed that PPO of spinach is enriched in the fraction containing grana lamellae obtained by discontinuous sucrose-density gradient centrifugation. Histochemical assays are based on the oxidation of ortho-diphenol by PPO to form brown pigments. The brown pigment can be observed under a microscope. Parish\(^{16}\) and Vaughn and Duke\(^{17}\) showed that PPOs of spinach beet and Sorghum are in the chloroplasts. Ohl and Mueller\(^{18}\) showed that thylakoids of plastids in a suspension culture of carrot are stained with L-hydroxyphenylalanine by the action of PPO. Sherman et al.\(^{19}\) surveyed histochemically the distribution of PPO and showed that thylakoids of such genera of angiosperms as *Anacharis densa*, *Lemna*, *Artemisia*, *Cucurbita*, and *Vigna* are uniformly stained by hydroxyphenylalanine. Changes in plastid can result in the complete loss of PPO activity. Vaughn and Duke\(^{20}\) showed that PPO activity is completely absent in all plastids from sensitive plants grown in a solution of tentoxin, which specifically affect plastids, causing chlorosis.

PPO cDNAs of such plants as *Vicia faba*,\(^{21} \) *tomato*,\(^{22} \) *23* *Phytolaca americana*,\(^{24}\) spinach,\(^{25}\) and apple\(^{26}\) have been cloned. The deduced amino acid sequences showed that PPO had a transit peptide sequence for plastids and thylakoids.\(^{27}\) Genomic DNA of PPO cloned from tomato has similar sequence for its transit peptide.\(^{28}\) We also cloned two genomic DNAs of apple PPO, which had sequences similar to the transit peptide for plastids and thylakoids (unpublished results). Sommer et al.\(^{29}\) showed that a precursor of tomato PPO is routed to thylakoid lumens in two steps by *in vitro* transcription, translation, and importation.

These results suggest that most apple PPO is in plastids, as is the PPO of other plants. Harel et al.\(^{3,4}\) obtained a crude chloroplast fraction and showed that PPO activity is recovered, when the fraction is treated with detergent. Our crude plastid fraction seemed to contain disintegrated organelles and the mitochondrion fraction seemed contain some plastids.

Immunohistochemical assays are specific, sensitive, and simple. The organelles detected immunochemically as containing PPO in immature fruit or suspension culture seemed to be plastids, from the results of density gradient centrifugation.

When plastids were further purified from the crude plastids from mature apple fruits, the specific activities of PPO and marker enzymes for plastids were highest of all, although catalase activity also was present. Contamination by catalase has been observed in purified plastid fraction\(^9\) and a part of the catalase activity may be endogenous catalase in plastids.\(^{11,11}\) Maturity of apples may make purification of plastids difficult.

The finding of high PPO activity in the soluble fraction of some apples might be an artifact arising during the preparation of the crude plastid fraction. PPO might become soluble. Marques et al.\(^{30}\) showed that there are two isoforms of PPO in apple fruits and that the 64 kDa isoform is proteolyzed into the other isoform (42 kDa). Our results showing high PPO activity in soluble fractions with decomposed fragments of plastid PPO were similar. Fraignier et al.\(^{31}\) showed such proteolysis of PPO in *Prunus* fruits. We found before that some denaturation of PPO occurs during apple ripening.\(^{7}\) Some PPO in plastids of apples probably becomes soluble during ripening and storage.

In conclusion, most apple PPO is located in plastids, as are PPOs in other plants. During ripening and storage, the PPO may be solubilized and proteolyzed.

**References**


