# Immunochemical and Immunohistochemical Study of Apple Chlorogenic Acid Oxidase

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Mouse antibody was raised against chlorogenic acid oxidase from apple (*Malus pumila* cv. Fuji). This antiserum was used for immunochemical characterization of the enzyme of apple and other plants and then for the immunocytolocalization of the enzyme in apple fruit to clarify the relationship between the distribution of the enzyme and browning. Western blot analysis revealed that the antibody recognized only the purified enzyme from apple fruit, with a molecular weight of 65 000. All of the apple cultivars showed the same band as the purified enzyme of Fuji. When the cross-reactivity of polyphenol oxidase between apple and other plants was examined, the antiserum only recognized the enzyme of pear. The browning of the apple fruit sections was predominantly observed around the core. Likewise, chlorogenic acid oxidase was also localized mainly near the core as shown by the nitrocellulose tissue print method using immunostaining.

## INTRODUCTION

Polyphenol oxidase (*o*-diphenol:oxygen oxidoreductase, EC 1.10.3.1, PPO) is largely responsible for enzymatic browning in fruits and vegetables, which is often a commercially undesirable reaction in foods. PPO has been frequently studied with regard to its purification, molecular weight, and substrate specificity (Harel *et al.*, 1965; Flurkey, 1980; Janoviz-Klapp *et al.*, 1990; Oda *et al.*, 1989). Little information is available concerning the histochemical localization and the immunological relationship among PPOs from different species.

The PPO of spinach leaves consists of chloroplastic isoenzymes of stroma and membrane forms. The isoenzymes were shown to be immunologically related by double diffusion and immunoprecipitation analysis (Lieberei *et al.*, 1981). Flurkey (1985) identified a specific *in vitro* translation product corresponding to broad bean PPO using polyclonal antibodies against broad bean PPO. The PPOs of eight different plant species also cross-reacted with the antibody against the broad bean enzyme in Ouchterlony double-diffusion analysis (Flurkey, 1986). In addition, a monoclonal antibody against the broad bean PPO was prepared. Both the polyclonal and monoclonal antibodies recognized similar enzyme forms in broad bean and mung bean, but only the polyclonal antibodies identified PPO of other plants (Lanker *et al.*, 1988). In this way, antibodies can be very useful for identifying isozyme forms or for determining the cross-reactivity among different plant species. Very few immunohistochemical localization studies have been performed on PPO. Only mushroom tryosinase has been studied immunohistochemically, and localization of the enzyme was shown (Moore *et al.*, 1988).

Apple is one of the most popular fruits for which enzymatic browning is important in processing, and the phenomenon of browning is experienced in usual food lives. We have previously reported the purification of chlorogenic acid oxidase, which is a major PPO in apple (Murata *et al.*, 1992). In this paper, we describe the preparation of polyclonal antibodies against the purified enzyme and the cross-reactivity of the enzyme between apple cultivars or other plant species. Furthermore, immunohistochemical localization of chlorogenic acid oxidase was accomplished using the antibody to clarify the relationship between the distribution of the enzyme and browning.

## MATERIALS AND METHODS

**Plant Materials.** The fruits of apple (*Malus pumila* cv. Fuji, Kōgyoku, Mutsu, Jona Gold, and Ōrin), eggplant (*Solanum melongena*), banana (*Musa carendishii* L.), and pear (*Pyrus serotina*) and leaves of broad bean (*Vicia faba*), spinach (*Spinacia oleracea* L.), and Lettuce (*Lactuca sativa* L.) were obtained from a local market in Tokyo.

**Extraction of PPO.** Peeled apple fruits were homogenized in 10 volumes of cold acetone, and the precipitate was washed with acetone until the supernatant became colorless. The precipitate was dried *in vacuo* at 4 °C. Crude extracts of apple were prepared by homogenizing 0.5 g of acetone powder in a total of 40 mL of 1% Triton X-100 in 20 mM phosphate buffer (pH 7.2) with a mortar and pestle. The homogenates were filtered and centrifuged at 10000*g* for 20 min. The supernatant was used as a crude enzyme extract.

PPOs of eggplant, banana, and pear were partially purified according to the methods of Fujita and Tono (1988), Galeazzi *et al.* (1981), and Tono *et al.* (1986), respectively, with some modification.

Fruits of eggplant (1.16 kg) were homogenized in 2 volumes of 0.1 M sodium phosphate buffer (pH 7.2), and the homogenate was filtered through four layers of cotton gauze and centrifuged at 8000*g* for 20 min. The supernatant was purified by ammonium sulfate precipitation (0–80%) and a DEAE-Toyopearl column (2.6 cm i.d. × 7.5 cm, Toso, Tokyo), which was equilibrated with 10 mM phosphate buffer (pH 7.2). The crude enzyme was obtained by eluting the column with a linear phosphate buffer gradient from 10 to 300 mM (pH 7.2).

Fruits of banana (748 g) were homogenized in 2 volumes of 0.2 M phosphate buffer (pH 7.0) containing 1% Polyclar AT and 1% Triton X-100 (Galeazzi *et al.*, 1981) and centrifuged at 10000*g*

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for 15 min. The acetone precipitate obtained by the addition of 3 volumes of cold acetone to the supernatant was re-extracted with 0.25% Triton X-100 in 0.2 M phosphate buffer (pH 7.2). The supernatant was dialyzed against 10 mM phosphate buffer (pH 7.2) and then applied to a DEAE-Toyopearl column (2.6 cm i.d. × 19 cm), which was equilibrated with 10 mM phosphate buffer (pH 7.2). The enzyme was eluted from the column with a linear phosphate buffer gradient from 0.01 to 0.3 M (pH 7.2).

Fruits of pear (291 g) were homogenized in 2 volumes of 0.1 M phosphate buffer (pH 7.2) and centrifuged at 10000*g* for 30 min. The supernatant was dialyzed against 10 mM phosphate buffer (pH 7.2) and applied to a DEAE-Toyopearl column (2.6 cm i.d. × 30 cm), which was equilibrated with 10 mM phosphate buffer. The enzyme was eluted from the column with 0.3 M phosphate buffer (pH 7.2).

Crude extracts of broad bean, spinach, and lettuce leaves were prepared according to the method of Flurkey (1986). Three grams of leaf was mixed with 25 mL of 20 mM phosphate buffer (pH 6.0) in a mortar and pestle. The homogenate was filtered through four layers of cotton gauze.

**Purification of Chlorogenic Acid Oxidase of Apple.** Apple chlorogenic acid oxidase was purified to homogeneity according to the method described previously (Murata *et al.*, 1992). The purified and crude enzymes eluted from the first DEAE-Toyopearl column in the isolation procedure were used for the immunochemical studies.

**Preparation of Antibody.** For immunization, six ddY mice (5 weeks old, male) were intraperitoneally injected with a dose of the purified enzyme (20 μg) emulsified (1:1) with Freund's complete adjuvant (Difco, Detroit). After 3 weeks, the booster injections were intraperitoneally performed with 5 μg of purified enzyme. The mice were boosted totally four times every 13 days with the same amount of the enzyme. After 59 days from the first injection, the mice were intraperitoneally injected with 0.1 mL of Ehrlich's ascites carcinoma cells, and after 10 days serum and ascites fluid were collected. Antiserum was frozen with 0.1% sodium azide added and was used in all of the experiments without further purification.

**Enzyme Activity and Protein Assay.** Chlorogenic acid oxidase activity was measured spectrophotometrically at 325 nm to detect the decrease of chlorogenic acid (Fujita *et al.*, 1988). A decrease in absorbance of 0.1 per minute at 30 °C was defined as 1 unit of chlorogenic acid oxidase activity. Pyrocatechol oxidase activity was assayed according to the method of Flurkey (1986) in the presence or absence of 0.05% sodium dodecyl sulfate (SDS). The absorbance at 410 nm of the raction mixture was measured.

Protein was assayed according to the Bradford method with bovine serum albumin as standard (Bradford, 1976).

**Immunological Methods.** Ouchterlony double-diffusion analysis was performed in 1% agarose gel prepared in phosphate-buffered saline consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, and 1.5 mM $KH_2PO_4$ (PBS, pH 7.2) containing 0.02% sodium azide. About 2 mL of the melted agar was poured on a microscope slide (25 × 75 mm). The thickness of the agar was about 1 mm. The diameter of a well was 2 mm, and the distance between the centers of the wells was 4 mm. Three microliters of each antiserum or enzyme solution was added to the wells, and the gel was left at room temperature overnight. For enzyme activity staining, the precipitates in the gel were stained with 0.8 mM chlorogenic acid and 0.8 mM (+)-catechin in McIlvaine buffer prepared by mixing 0.1 M citric acid with 0.2 M $Na_2HPO_4$ (pH 4.0) after the gel was washed in water.

Indirect microplate enzyme-linked immunosorbent assay (ELISA) was performed using horseradish peroxidase-labeled secondary antibody. The purified chlorogenic acid oxidase (0.5 μg/well in 50 μL of PBS) was added in each well of a polystyrene microplate (Nunc, Maxisorp, Røskilde, Denmark) overnight at 4 °C. Following three washes with PBS containing 0.05% Tween 20, 350 μL/well of blocking solution (2% bovine serum albumin in PBS) was added for 2 h at room temperature. Microwells were washed three times, and then 50 μL of diluted antiserum in PBS was added and incubated at 4 °C overnight. The plate was then washed three times, and 50 μL of anti-mouse IgG peroxidase conjugate (Dakopatts, Denmark), diluted 1:2000 in PBS was added to each well. The plate was incubated for 60 min at 4 °C and then washed as before. Finally, 200 μL of substrate solution (4 mM *o*-phenylenediamine, 0.004% $H_2O_2$ in 50 mM sodium phosphate–20 mM citric acid buffer, pH 5.0) was added. The color change in each well was measured spectrophotometrically at 415 nm.

Immunoprecipitation was carried out by incubating the enzyme solution with various concentrations of antiserum overnight at 4 °C. At first the enzyme activity of the reaction mixture was measured. Then the reaction mixture was centrifuged at 10000*g* for 30 min, and the enzyme activity of the supernatant was measured. Because antiserum was stored in 1% sodium azide and 0.9% sodium chloride, which inhibit the enzyme activity, the antiserum was applied to a Sephadex G-25 column (1.2 cm i.d. × 20 cm, Pharmacia, Uppsala, Sweden) and developed with 20 mM phosphate buffer (pH 7.2) to remove the inhibitors before use.

**Electrophoresis and Western Blotting.** Polyacrylamide gel electrophoresis (PAGE) and SDS–PAGE were performed with Phastsystem (Pharmacia) using Phastgel (8–25% gradient). Samples were denatured by incubation in boiling water for 3 min with 2% SDS and 0.05 M β-mercaptoethanol for SDS–PAGE, and the gel was stained with Coomassie Brilliant Blue R-250. In native PAGE, the gel was also stained for enzyme activity with chlorogenic acid and (+)-catechin in McIlvaine buffer (pH 4.0).

Samples on PAGE and SDS–PAGE were thermoblotted onto nitrocellulose membranes (0.45 μm, Schleicher and Schuell, Keene, NH) for 30 min at 70 °C.

At first, nitrocellulose blots were blocked with 5% skim milk in PBS, for 30 min. Primary antibody against apple chlorogenic acid oxidase was diluted 1:2000 in blocking solution, and incubation was continued overnight at 4 °C. Anti-mouse IgG peroxidase conjugate was diluted 1:1000, and incubation was continued for 1 h. The detection of peroxidase on protein blots was performed by addition of 0.02% diaminobenzidine and 0.03% chloronaphthol and 0.2 μL/mL $H_2O_2$ as a substrate.

**Nitrocellulose Tissue Prints.** Nitrocellulose tissue prints were performed following the method of Reid *et al.* (1990) with some modification. Nitrocellulose was soaked in 3% Triton X-100 in 50 mM phosphate buffer (pH 7.2), which was the best blotting condition, and wiped on filter paper. Apples were cut vertically and blotted directly onto the membrane by finger pressure for 1 min.

The detection of the antigen was performed as described for Western blotting. The membrane was blocked with 5% skim milk in 20 mM Tris-buffered saline (pH 7.5). The primary antibody was diluted 1:100, and the anti-mouse IgG peroxidase conjugate was diluted 1:100 in 20 mM Tris-buffered saline (pH 7.5) containing 0.05% Tween 20.

**Immunohistochemistry.** The apple tissue section (about 3 × 4 mm) around the core was transferred to a fixation medium containing 10% (v/v) formaldehyde in PBS and left overnight at room temperature. After washing with PBS, the fixed tissue section was dehydrated through an ethanol series [2.5, 5, 10, 20, 30, 40, 60, 70, 80, 90, 95, 99.5% (v/v)] for 1 h per step and then 100% ethanol overnight. The sections were embedded using a JB-4 embedding kit (Polyscience, Warrington, PA) following the manufacturer's instructions. Sections, 20 μm thick, were cut with a microtome and mounted on glass slides.

The antigen was stained with anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (Dakopatts, Denmark). The primary antibody against apple chlorogenic acid oxidase was diluted 1:100 in PBS and incubated for 5 h at room temperature. Following three washes with PBS, some drops of anti-mouse IgG FITC conjugate diluted 1:20 in PBS were added to each section. The sections were washed with PBS before observation with a fluorescence microscope (Olympus BH-2, Tokyo).

## RESULTS

**Immunological Properties of Anti-Chlorogenic Acid Oxidase of Apple.** Seventy days after the first injection, antiserum was obtained from serum and ascites fluid. The titer of the antiserum was determined by ELISA using the purified enzyme and was found to be 1:10000,

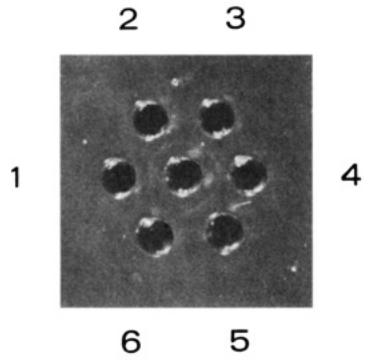


**Figure 1.** Ouchterlony double-diffusion analysis of the antiserum to chlorogenic acid oxidase of apple. (Center well) antiserum; (wells 1, 3, 5) purified enzyme; (well 2) crude extract from acetone powder; (well 4) crude enzyme after the first DEAE-Toyopearl column chromatography; (well 6) PBS. The crude enzyme was prepared according to the method of Murata *et al.* (1992).

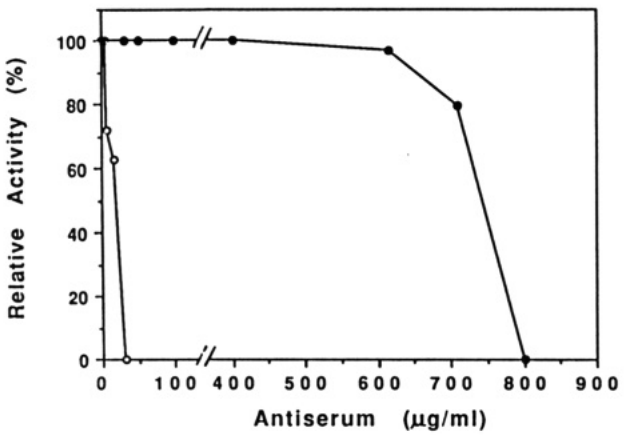


**Figure 2.** Immunotitration curve of the antiserum against the activity of chlorogenic acid oxidase. Purified chlorogenic acid oxidase (5.5 units/mL) was incubated at 4 °C overnight with the antiserum. The enzyme activity remaining was measured spectrophotometrically.

suggesting high specificity against purified enzyme. Figure 1 shows an Ouchterlony double-diffusion analysis. It is obvious that only one immunoprecipitation band was formed with purified enzyme. Furthermore, the antiserum also cross-reacted with the crude enzyme. All precipitation lines were fused, and no single- or double-spur reaction occurred.

The immunotitration curve for the purified enzyme shows that the enzyme activity in the solution remained high even with a high antibody concentration, 700 μg/mL. After centrifugation, however, there was a decrease in enzyme activity in the supernatant at low antibody concentrations, and the enzyme activity was completely lost with 40 μg/mL of antiserum (Figure 2). These results showed that at low antibody concentrations, antigen–antibody complexes were formed, but that the complex retained enzyme activity even at high antibody concentrations. In Ouchterlony double-diffusion analysis, the gel was enzymatically stained with polyphenols. As a result, the precipitation band turned brown (Figure 3). This also suggests that the enzyme in the precipitation band still had enzyme activity despite forming a high molecular complex with the antibody. Therefore, the antibody may combine with sites other than the active site of the purified enzyme.

**Western Blot Analysis of Crude Enzyme of Apple.** The purified and crude enzymes of apple were analyzed by native PAGE and SDS–PAGE with protein and

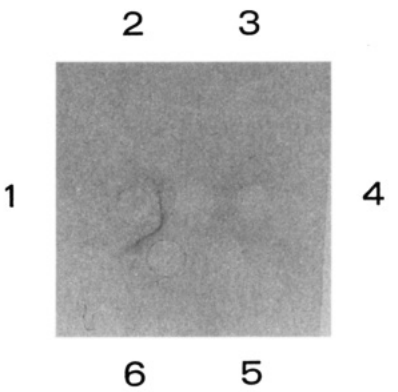


**Figure 3.** Staining for enzyme activity in Ouchterlony double-diffusion analysis of antiserum and chlorogenic acid oxidase of apple. After immunoprecipitation was observed, the gel was stained for enzyme activity. (Center well) purified enzyme; (well 1) antiserum; (well 2) crude extract from acetone powder containing 1% SDS; (well 3) control serum; (well 4) PBS; (well 5) control serum; (well 6) crude extract from acetone powder.

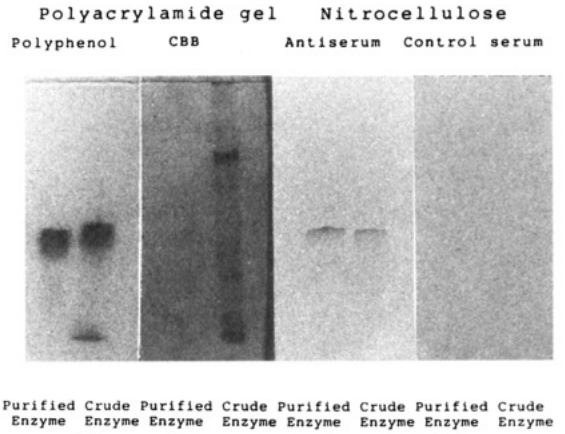


**Figure 4.** Western blot analysis of purified chlorogenic acid oxidase and crude extract of apple following native PAGE. The enzyme solution was electrophoresed on polyacrylamide gels (8–25% gradients). The gel was stained with CBB or polyphenols. The crude enzyme was prepared by the first chromatography through a DEAE-Toyopearl column.

enzymatic activity staining with polyphenols and then studied by Western blot analysis.

In native PAGE, the purified enzyme showed a brown band and the crude enzyme showed the same major brown band and another faint band upon staining with chlorogenic acid and (+)-catechin (Figure 4). Only one band with the same mobility in both the purified and crude enzymes was immunologically stained on nitrocellulose. The blotted band on the membrane corresponded to the brown band and protein band on the gel. The antiserum did not recognize the faint isoenzyme band in the crude enzyme. No immunostaining was present in control blots treated with nonimmune mouse serum.

Western blot analysis following SDS–PAGE also showed only a single band with both the purified enzyme and crude enzyme preparations (Figure 5). By calibration with the molecular weight markers, the molecular mass of the band was estimated to be 65 000, which was in agreement with the molecular weight of the purified enzyme (Murata *et al.*, 1992). Nonimmune serum did not give any band.

**Cross-Reactivity of the Antibody to PPO of Apple.** The differences between PPOs from cultivars of apple (Kōgyoku, Mutsu, Jona Gold, Ōrin, and Fuji) were studied using the antibody against the purified enzyme of Fuji. All of the crude extracts showed chlorogenic acid oxidase activity; Fuji showed the highest activity (23.6 units/g of

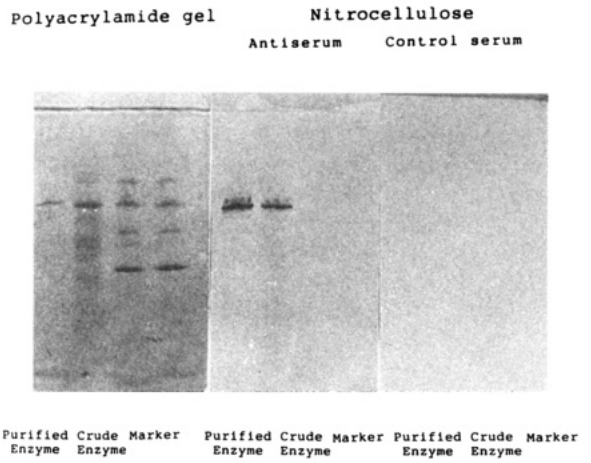


**Figure 5.** Western blot analysis of purified chlorogenic acid oxidase and crude extract of apple following SDS–PAGE. The enzyme solution was denatured and electrophoresed on SDS–polyacrylamide gels (8–25% gradients). The crude enzyme was prepared by the first chromatography through a DEAE-Toyopearl column.

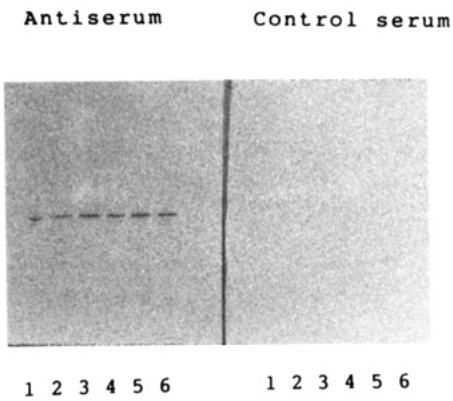


**Figure 6.** Western blot analysis of chlorogenic acid oxidase in crude extracts of some apple cultivars. The enzyme solution was denatured and electrophoresed on SDS–polyacrylamide gels (8–25% gradients). (Lane 1) purified enzyme (Fuji); (lanes 2–6) crude enzyme from acetone powder; (lane 2) Kōgyoku; (lane 3) Mutsu; (lane 4) Jona Gold; (lane 5) Ōrin; (lane 6) Fuji.

tissue), whereas Kōgyoku had the lowest activity (5.6 units/g of tissue). The crude extracts of each cultivar showed the same band, which is also the same as the band of the purified enzyme from Fuji, in Western blots (Figure 6). Therefore, it appears as if the five cultivars of apple contain the same chlorogenic acid oxidase.

**Cross-Reactivity between Apple PPO and PPOs of Other Plants Such as Eggplant, Banana, Pear, Broad Bean, Spinach, and Lettuce.** Crude extracts of eggplant, banana, and pears had chlorogenic acid oxidase activity, while crude extract of leaves of broad bean, spinach, and lettuce did not contain chlorogenic acid oxidase activity but did contain pyrocatechol oxidase activity, which increased in the presence of SDS. These crude PPOs from leaves differed in substrate specificity from those in other crude enzyme preparations of fruits.

Among the PPOs of six plant species, only pear PPO cross-reacted with anti-apple chlorogenic acid oxidase antibody in Western blot analysis following SDS–PAGE (Figure 7). The immunostaining band of pear PPO was estimated to have a molecular weight of 57 000, which is close to that of purified PPO of pear (Tono *et al.*, 1986). PPOs from the other plant species gave no immunostaining band. In Ouchterlony double-diffusion analysis, the immunoprecipitation band between antiserum and PPO of pear was fused with that formed between the antiserum

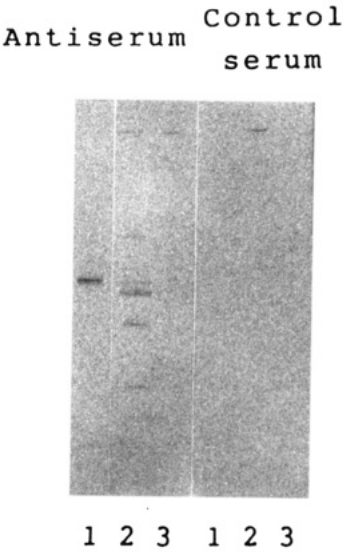


**Figure 7.** Cross-reactivity of the apple anti-chlorogenic acid oxidase antibody to PPO from other plants as determined by western blot analysis. The enzyme solution was denatured and electrophoresed on SDS–polyacrylamide gels (8–25% gradients). (Lane 1) purified chlorogenic acid oxidase from apple; (lane 2) PPO from pear; (lane 3) PPO from broad bean.

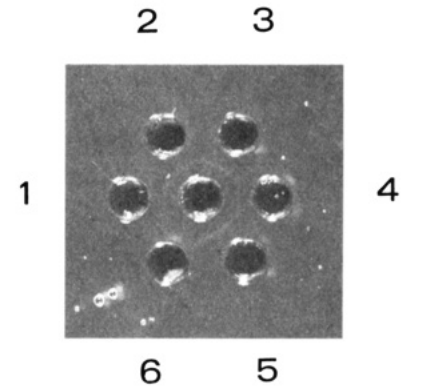


**Figure 8.** Cross-reactivity of apple anti-chlorogenic acid oxidase antibody to PPO from pear by Ouchterlony double-diffusion analysis. (Center well) antiserum; (wells 1, 3, 5) purified chlorogenic acid oxidase from apple; (well 2) PPO from pear; (well 4) crude enzyme of apple from the first DEAE-Toyopearl column chromatography; (well 6) PBS.

and chlorogenic acid oxidase from apple (Figure 8). Thus, the chlorogenic acid oxidase of apple and PPO of pears appear to be serologically related.

**Histological Localization of Chlorogenic Acid Oxidase.** The nitrocellulose tissue print method (Reid *et al.*, 1990) was adapted to detect simply and quickly the histological localization of chlorogenic acid oxidase by using an antibody. As the purified enzyme was extracted with Triton X-100 from a crude plastid fraction, the blotting efficiency of the enzyme was increased by treatment of the membrane with 3% Triton X-100. The antibody strongly reacted with the part at the core and, second, the part near the skin on the nitrocellulose membrane (Figure 9). The immunostaining of the membrane suggests that chlorogenic acid oxidase is not distributed uniformly but is mainly localized at the core in apple fruit and secondarily near the skin. Tissue prints were also stained with amido black, and the protein distribution was relatively homogeneous in the apple tissue.

The browning was not uniformly distributed in the cross section of apple fruit, being most intense at the core and secondarily near the skin (Figure 10). Comparing the localization of the enzyme with the browning shows that

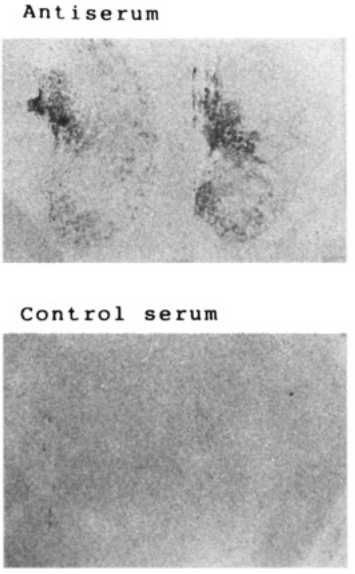


**Figure 9.** Distribution of chlorogenic acid oxidase in apple tissue visualized by nitrocellulose tissue print method. The tissue was printed on nitrocellulose, and the paper was stained by the apple chlorogenic acid oxidase antiserum and peroxidase conjugated secondary antibody.

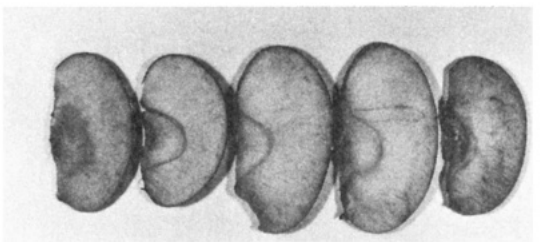


**Figure 10.** Browning of apple flesh. The tissue was left overnight at room temperature.

the browning of the apple section corresponds to the distribution of the chlorogenic acid oxidase, although other factors such as substrate concentration and condition of cells might be also important near the skin.

**Immunocytochemistry of Chlorogenic Acid Oxidase in Apple Cell.** The cytochemical localization of chlorogenic acid oxidase in apple cells was examined using immunofluorescence. The immunofluorescence was intense near the periphery of the cell (Figure 11). The apple fruit plays a role in the storage of materials, and it is known that the cell is occupied by vacuole. Plastid, in which chlorogenic acid oxidase exists, is suggested to be localized near the periphery of the cell.

## DISCUSSION

Chlorogenic acid and catechin are major polyphenols in apple. However, chlorogenic acid oxidase has not been studied in apple. We have purified chlorogenic acid oxidase to homogenity from apple fruit (Murata *et al.*, 1992). In this paper, we described some immunochemical and immunohistochemical studies of apple chlorogenic acid oxidase.

The antiserum against purified apple chlorogenic acid oxidase was specific for the purified enzyme (Figure 4 and 5), and five cultivars of apple have the same enzyme (Figure 6).

Flurkey (1986) studied the cross-reactivity of PPO from leaves of eight plant species using polyclonal antibodies

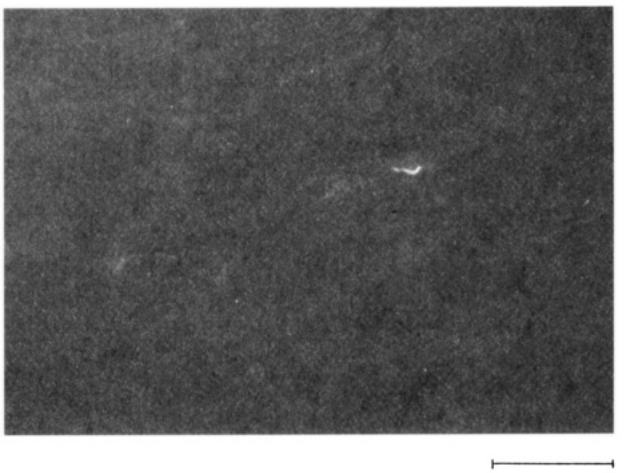

**Figure 11.** Immunocytochemical localization of chlorogenic acid oxidase in apple cell. The section was stained by indirect immunofluoresence method. Bar, 0.1 mm.

against broad bean PPO using pyrocatechol for the PPO assay. In this study PPOs of broad bean, spinach, and lettuce were found to be serologically related by Ouchterlony double-diffusion analysis. In our studies anti-chlorogenic acid oxidase of apple did not recognize these PPOs from leaves. This result may be because the crude extracts of broad bean, spinach, and lettuce did not contain chlorogenic acid oxidase but contained pyrocatechol oxidase. In contrast, pear PPO, which shows chlorogenic acid oxidase, cross-reacted with the antiserum. This result could be due to the fact that pear and apple belong to the same family.

Histochemical localization of PPO of mushroom was studied by Moore *et al.* (1988). There have been no studies of the histochemical localization of chlorogenic acid oxidase of fruit originated from plants. We showed that both chlorogenic acid oxidase and the browning reaction are localized intensely near the core and secondarily near the skin in apple sections using immunostaining. We conclude that the browning of apple fruit near the core corresponds with the localization of chlorogenic acid oxidase activity.

## ACKNOWLEDGMENT

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## LITERATURE CITED

Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

Flurkey, W. H. Purification of peach polyphenol oxidase in the presence of added protease inhibitors. *J. Food Biochem.* **1980**, *4*, 29–41.

Flurkey, W. H. *In vitro* biosynthesis of *Vicia faba* polyphenoloxidase. *Plant Physiol.* **1985**, *79*, 564–567.

Flurkey, W. H. Polyphenoloxidase in higher plants. *Plant Physiol.* **1986**, *81*, 614–618.

Fujita, S.; Tono, T. Purification and some properties of polyphenoloxidase in eggplant (*Solanum melongena*). *J. Sci. Food Agric.* **1988**, *46*, 115–123.

Galeazzi, A. M.; Sgarbieri, V. C.; Constantinides, S. M. Isolation, purification and physicochemical characterization of polyphenoloxidases (PPO) from Dwarf variety of banana (*Musa cavendishii*, L). *J. Food Sci.* **1981**, *46*, 150–155.

Harel, E.; Mayer, A. M.; Shain, Y. Purification and multiplicity of catechol oxidase from apple chloroplasts. *Phytochemistry* **1965**, *4*, 783–790.

Janovitz-Klapp, A. H.; Richard, F. C.; Groupy, P. Y.; Nicolas, J. J. Kinetics studies on apple polyphenol oxidase. *J. Agric. Food Chem.* **1990**, *38*, 1437–1441.

Lanker, T.; Flurkey, W. H.; Hughes, J. P. Cross-reactivity of polyphenoloxidase in higher plants. *Phytochemistry* **1988**, *27*, 3731–3734.

Lieberei, R.; Biehl, B.; Voigt, J. Serological studies on phenolase from spinach leaves. *Phytochemistry* **1981**, *20*, 2109–2116.

Moore, B. M.; Kang, B.; Flurkey, W. H. Histochemical and immunochemical localization of tyrosinase in whole tissue sections of mushroom. *Phytochemistry* **1988**, *27*, 3735–3737.

Murata, M.; Kurokami, C.; Homma, S. Purification and some properties of chlorogenic acid oxidase from apple (*Malus pumila*). *Biosci., Biotechnol. Biochem.* **1992**, *56*, 1705–1710.

Oda, Y.; Kato, H.; Isoda, Y.; Takahashi, N.; Yamamoto, T.; Takada, T.; Kudo, S. Purification and properties of phenoloxidase from spinach leaves. *Agric. Biol. Chem.* **1989**, *53*, 2053–2061.

Reid, P. D.; del Campillo, E.; Lewis, N. L. Anatomical changes and immunolocalization of cellulase during abscission as observed on nitrocellulose tissue prints. *Plant Physiol.* **1990**, *93*, 160–165.

Tono, T.; Fujita, S.; Kawasaki, H.; Li, Z. Purification and high L-epicatechinoxidase activity in polyphenoloxidase of Japanese pears. *Nippon Nogeikagaku Kaishi* **1986**, *60*, 705–712.

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