Subcellular Localization of Lipoxygenase-1 and -2 in Germinating Soybean Seeds and Seedlings[1]

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The subcellular localization of lipoxygenase-1 and -2 in the cotyledons of germinating soybean seeds [*Glycine max* (L.) Merr. AmSoy] and seedlings was investigated by direct labeling with immunogold complexes. Both lipoxygenase-1 and -2 were localized in the cytoplasm of all cotyledon cells. No gold labeling was observed on mitochondria, glyoxysomes, protein bodies, lipid bodies, or other organelles.

KEY WORDS: Direct immunogold labeling, germination, lipoxygenase-1, lipoxygenase-2, localization, soybean, subcellular organelles.

Lipoxygenase (E.C.1.13.11.12; linoleate: oxygen oxidoreductase) is a dioxygenase that contains nonheme iron. It catalyzes the oxidation of polyunsaturated fatty acids having a *cis,cis*1,4-pentadiene system to produce conjugated hydroperoxydiene derivatives through the insertion of molecular oxygen into the substrate. These primary products are converted into secondary products, such as alcohols, aldehydes, ketones, and epoxy compounds, by enzymatic and nonenzymatic processes, which are responsible for the development of off-flavors during the processing of certain plant food-stuffs, particularly soybeans (1-3). Seeds of soybean [*Glycine max* (L.) Merr.] contain significant amounts of lipoxygenase-1 and -2 (1.4 and 2.8 mg/g dry weight, respectively) (4). The properties of lipoxygenase and the methods for its purification have been studied extensively, and the existence of multiple lipoxygenase in soybeans also is well established (5-7).

For better understanding of the action, distribution, and physiological function of lipoxygenase in plants, the exact cellular and subcellular localization of lipoxygenase has been emphasized. Unfortunately, evidence for the subcellular localization of lipoxygenase is insufficient. Douillard and Bergeron (8) found that lipoxygenase activity was distributed in chloroplast stroma of young pea shoots, and Wardale and Galliard (9) reported that lipoxygenase in soybean seeds was localized in particle-free supernatant fractions.

An immunocytochemical technique was used to investigate the subcellular distribution of lipoxygenase in soybean cotyledons. Antibodies directed against lipoxygenase-1 and -2 were raised from goats. Antibodies absorbed onto gold particles (immunogold complexes) were used for the direct labeling of lipoxygenase-1 and -2 in the cotyledons of germinating soybean seeds and seedlings. The observations were made in three different tissues—epidermis, vascular bundle, and storage parenchyma—to compare the potential lipoxygenase metabolic differences between the component cells within the cotyledons.

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EXPERIMENTAL PROCEDURES

Seed germination. Soybean seeds [*Glycine max* (L.) Merr. AmSoy] were obtained from the Department of Agronomy, Iowa State University (Ames, IA). Seeds were externally sterilized in 70% ethanol for 10 seconds, rinsed with running water for 10 min, and soaked for 50 min in oxygen-flushed, twice-distilled water. Seeds were then layered on wet filter paper on cotton wool in a plastic box with a lid. The box was placed in the dark at 28 ± 1°C. Cotyledons of soybean seeds were selected from 1 day (1d) through 7 days (7d) of germination and seedling growth.

Lipoxygenase assay. Lipoxygenase was determined polarographically by using a YSI oxygen Monitor Model 53 equipped with a Clark electrode (Yellow Spring Instruments, Yellow Springs, OH). Throughout this experiment, an aqueous linoleate substrate (10) was used. A stock solution was prepared by adding 0.1 mL of linoleic acid to 0.1 mL of Tween 20. To obtain a clear solution, 0.3 mL of NaOH was slowly added, and the dispersion was brought to a final volume of 25 mL with deinonized water. The stock substrate solution was diluted with 50 mM borax buffer (pH 9.0) and 50 mM Na phosphate buffer (pH 6.8) for lipoxygenase-1 and -2 assays, respectively, giving a final concentration of 2.57 mM linoleic acid.

Appropriate substrates (2.99 to 2.95 mL) were pipetted into a reaction chamber and oxygenated for 30 seconds. Enzyme solutions (10 to 50 uL) were added to the chamber and stirred with a magnetic stirrer. The initial velocity of the enzyme reaction was recorded. Values for oxygen solubility in the reaction medium were obtained from Chappell (11). One unit of lipoxygenase activity corresponds to the consumption of 1 mM of oxygen per minute.

Protein determination. Protein concentrations were determined by optical density at 280 nm and by the Lowry *et al.* method (12).

Tissue processing. Tissues were processed according to a modification of the method of Herman and Shannon (13). Cotyledons of germinated soybean seeds and seedlings were placed in 2% (v/v) glutaraldehyde-4% (w/v) paraformaldehyde in 0.1 M Na phosphate buffer (pH 7.4) and dehydrated for 1 hr each at 20°C in dimethylformide (DMF) by using 25%, 50%, 75%, and 100% (v/v) solutions. The dehydrated tissues were infiltrated with Lowicryl K 4M resin (Polysciences, Inc., Warrington, PA) containing 85% (w/w) monomer, 15% (w/w) crosslinker, and 0.5% (w/w) benzoin methyl ether. Infiltration was carried out at 20°C for 12 hr in a 1:1 mixture of resin and DMF, and then for two days in 100% resin. Aluminum foil was wrapped around the sample containers to minimize exposure to light.

Tissue samples were loaded into BEEM capsules (Ted Pella Co., Tustin, CA), filled with resin, and capped to preclude contact with air. The blocks were polymerized by illumination with longwave ultraviolet light for 24 hr

at 0°C and then for 24 hr at 20°C. Ultrathin sections of 60–85 nm were cut with glass knives on a Reichert Ultracut E ultramicrotome (C. Reichert AG, Wein, Austria) and mounted on 300-mesh nickle grids.

Enzyme purification and antibody production. Soybean lipoxygenase was isolated according to the methods of Yoon and Klein (14) and Axelrod *et al.* (6). Antisera to lipoxygenase-1 and -2 were raised in young female goats by subcutaneous injections of the enzyme fractions (2 mg/mL), which were mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Animals were injected at two-week intervals until a suitable titer was obtained. Before injection, blood samples were taken and saved for the control experiment of immunolabeling in electron microscopy.

Immunoglobulin G (Ig G) fractions of antisera were isolated by the precipitation of the sera with 0.15 M caprylic acid according to the method of Steinbuch and Audran (15). Anti-lipoxygenase-1 and -2 were affinity chromatographed over lipoxygenase-1 and -2 coupled to CNBr-activated sepharose 4B, respectively (16). Affinity-purified antibodies were analyzed by Ouchterlony double-diffusion tests (17).

Immunocytochemistry. Colloidal gold particles with an average diameter of 13–16 nm were obtained by reduction of tetrachloroauric acid with sodium citrate (18). Gold particles were adsorbed with anti-lipoxygenase-1 and -2 (immunogold complexes) according to Geoghegan and Ackerman (19) and Horisberger *et al.* (20).

Grids with sections were incubated in 0.05 M glycine in 20 mM phosphate buffer (pH 7.4) with 0.9% (w/v) NaCl (PBS) for 20 min to prevent nonspecific binding of immunogold complexes, then were washed three times with PBS buffer for 30 min. Immunogold complexes were diluted just before use until the color was faintly pink. Immunocytochemical labeling was accomplished by incubating the grids in the immunogold-complex solution for 60 min. The sections were washed three times for 10 min each with 20 mM PBS (pH 7.4), and three times with degassed, twice-distilled water. As a control, other grids were incubated in preimmune serum-gold complex solution prepared in the same manner as the immunogold complexes. After air drying, the sections were stained in 5% aqueous uranyl acetate at 37°C for 20 min. The grids were examined and photographed using a Hitachi HU-11C-1 transmission electron microscope (TEM) at 50 KV accelerating voltage.

RESULTS AND DISCUSSION

Morphology and lipoxygenase activity changes during germination and seedling growth. During germination of soybean seeds at 28 ± 1°C in the dark, the radicle emerged after the first day, and the hypocotyl began its elongation after the second day. Secondary roots appeared in four days, and cotyledons opened after the sixth day, revealing the expanding primary leaves.

Lipoxygenase-1 and -2/3 activities in AmSoy declined sharply after the second day, and after day 6 the activity of lipoxygenase decreased to a low level, the activity at pH 6.8 being somewhat higher than the activity at pH 9.0 (19 and 14% of maximal activity, respectively) (Fig. 1).

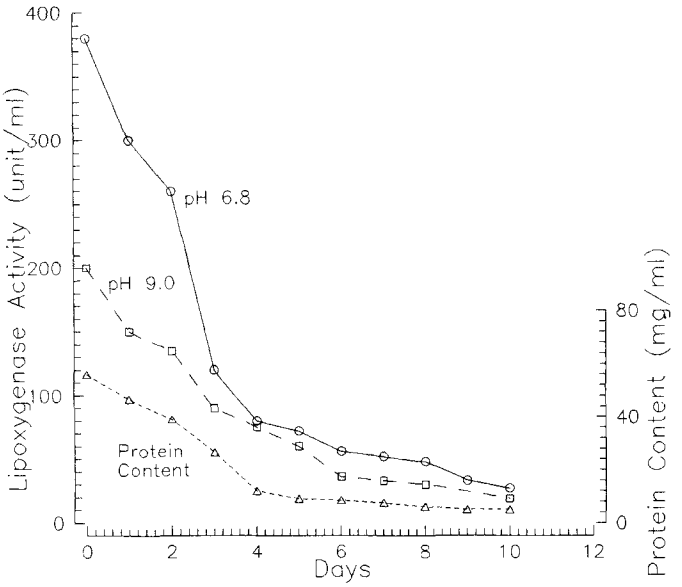

FIG. 1. Change of lipoxygenase-1 and -2 activities and protein content in AmSoy during germination and seedling growth.

Ultrastructure of cotyledons. Cotyledons of soybean are composed of an epidermis, storage parenchyma, and vascular tissues. Storage parenchyma tissue makes up most of the cotyledon and is surrounded by hypodermis and epidermis. Vascular bundles, surrounded by a parenchymatous bundle sheath, are situated along the boundary between adaxial and abaxial parenchyma.

When germination began, the cotyledon tissues were packed with protein and lipid bodies. Mobilization of these reserves started in the epidermis and vascular bundles. After three days of seedling growth, significant reductions of protein and lipid bodies were observed; concurrently, the number of starch grains, glyoxysomes, and mitochondria were increased. At later stages of seedling growth, cotyledon cells consisted of a large central vacuole surrounded by a small zone of cytoplasm.

Antisera specificity. Lipoxygenase-1 and -2 isolated from soybean were homogeneous on 7% PAGE (21). The antisera of lipoxygenase-1 and -2 raised in goats cross-reacted with soybean homogenates in double diffusion tests, resulting in a line of identity, demonstrating high specificity of the antisera preparation (Fig. 2).

Immunocytochemistry. Immunoelectron microscopy of ultrathin sections of soybean cotyledon showed that both lipoxygenase-1 and -2 were localized in the cytoplasm of all cotyledon cells (Figs. 3–6). No specific gold labeling was observed after incubation with nonimmune serum-gold complexes (Fig. 7). In storage parenchyma cells of day-old cotyledons, lipid and protein bodies fill the cytoplasm. After incubation with anti-lipoxygenase-1 and -2 Ig G, a high density of gold labels was found in regions of cytoplasm without lipid bodies (Figs. 3 and 4). In epidermis and vascular bundles of three-day-old cotyledons, both lipoxygenase-1 and -2 were randomly distributed throughout the cytoplasm. No association was found with protein bodies, lipid bodies, mitochondria, or other organelles (Figs. 5 and 6). After day 5, very weak gold labeling was observed in all cotyledon cells (Fig. 8), which indicates inactivation of lipoxygenase during seedling growth, as shown in Figure 1.

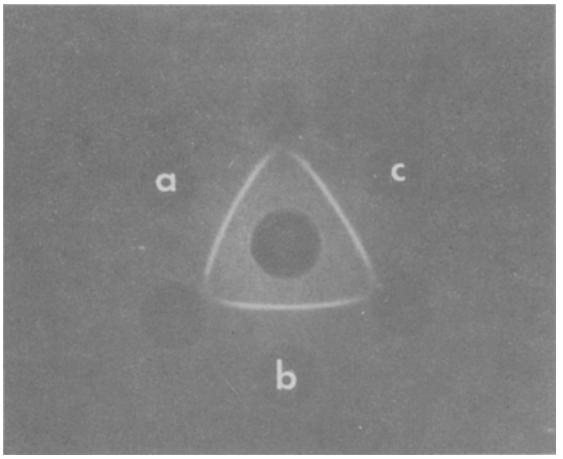

FIG. 2. Double-diffusion test of anti-lipoxygenase-1 and -2 against homogenate from soybean seeds. Central Well, homogenates from soybean seeds (20 mg/mL); Well a anti-lipoxygenase-1; and Wells b and c, anti-lipoxygenase-2 (2 mg/mL). The plate was stained with coomassie brilliant blue. A single precipitaton line occurred at the site of reaction between anti-lipoxygenase and homogenates from soybean seeds.

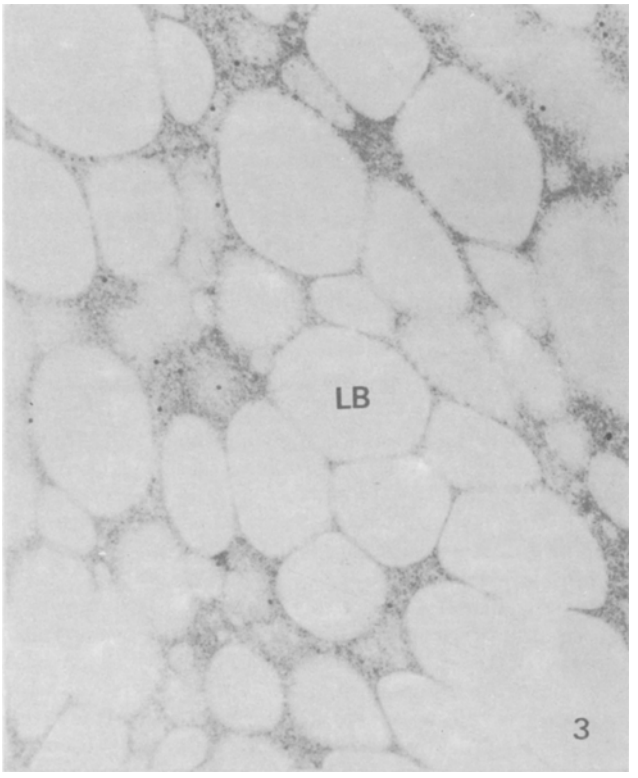

FIG. 3. Storage parenchyma cell of day-old cotyledon. After incubation with anti-lipoxygenase-1-Ig G-gold complexes, gold labels appear in cytoplasm without lipid bodies (LB); 30,100 x.

Similar results were obtained in potato tuber and soybean by Wardale and Galliard (9), where lipoxygenase activity was present in the particle-free supernatant after density gradient fractionation. They claimed that the activity of lipoxygenase would not sediment, possibly because of the destructive action of lipolytic acyl hydrolase on subcellular membranes during isolation.

Evidence for the subcellular distribution of lipoxyge-

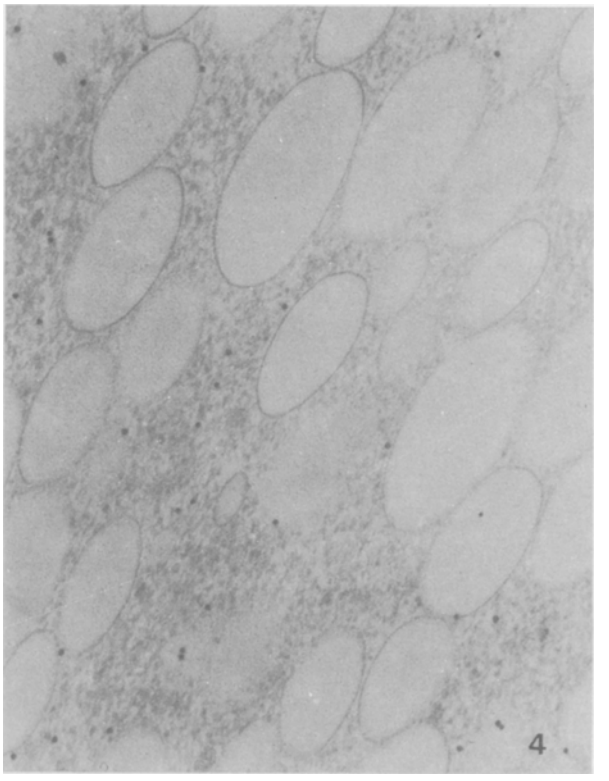

FIG. 4. Storage parenchyma cell of three-day-old cotyldeon. After incubation with anti-lipoxygenase-2-Ig G-gold complexes, gold labels appear in cytoplasm; 42,800 x.

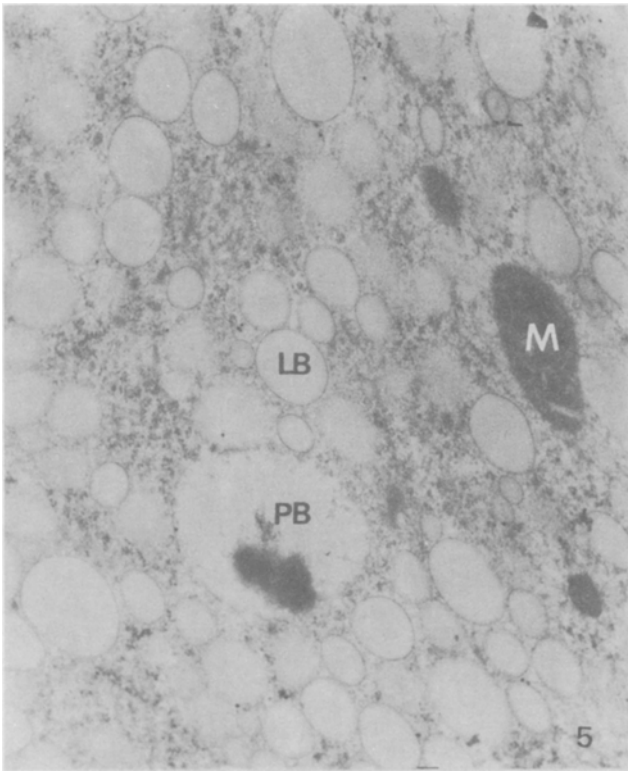

FIG. 5. Vascular bundle cell of three-day-old cotyledon. Numerous gold labels (anti-lipoxygenase-2-Ig G-gold complexes) are seen in cytoplasm. No gold label is seen in mitochondria (M), protein bodies (PB), or lipid bodies (LB); 59,300 x.

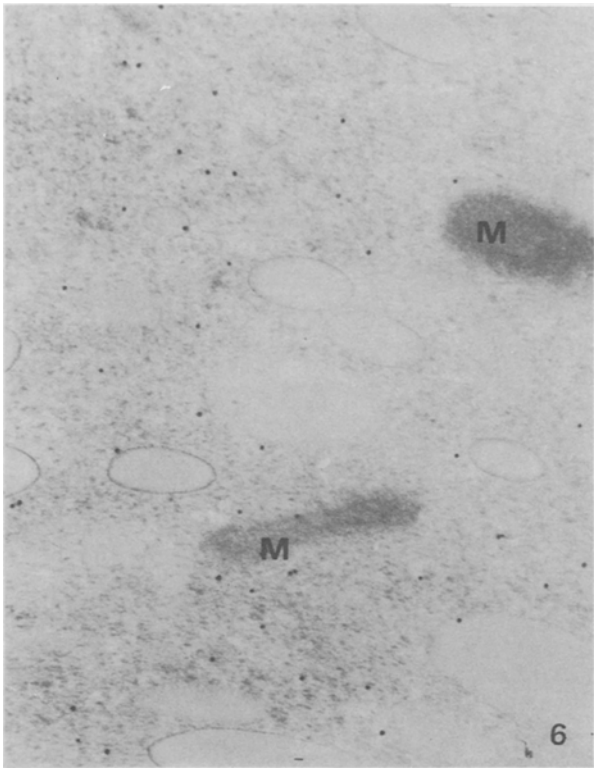

FIG. 6. Epidermal cell of three-day-old cotyledon. Gold labels (anti-lipoxygenase-1-Ig G-gold complexes) are seen in cytoplasm; 34,400 x.

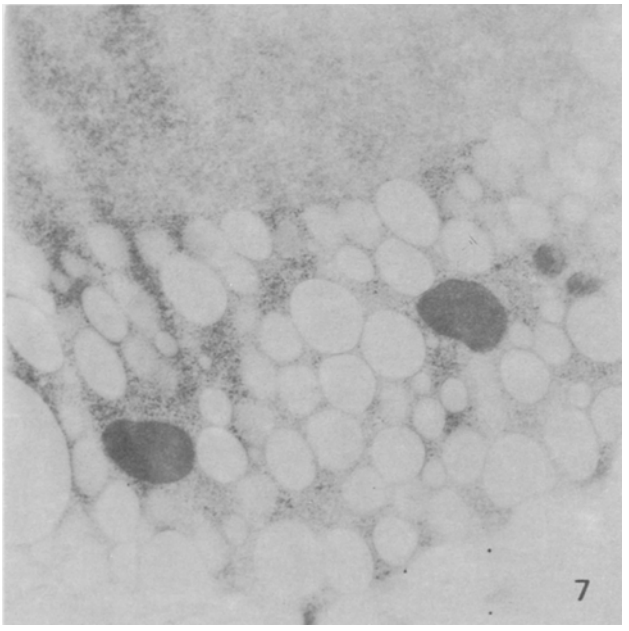

FIG. 7. Control incubated with nonimmune serum-gold complexes. No specific binding is seen in three-day-old cotyledon; 31,100 x.

nase by differential centrifugation and density gradient fractionation do not provide congruent information. Douillard and Bergeron found that lipoxygenase activity was distributed in the chloroplast lamellae of young wheat (22) and in chloroplast stroma of young pea shoots (8). High lipoxygenase activity was found in the intact

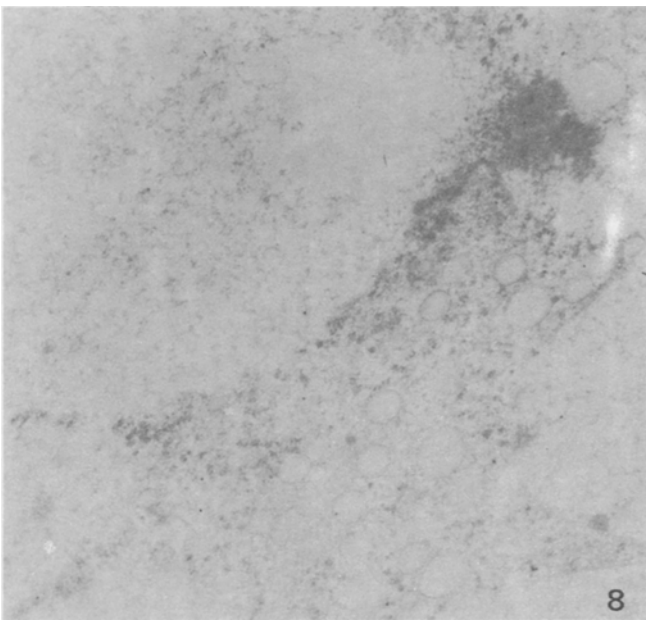

FIG. 8. Weak gold labeling is seen in cytoplasm after incubation with anti-lipoxygenase-2-Ig G-gold complexes in five-day-old cotyledon; 19,100 x.

proplasts and vacuoles from both peel and flesh tissues of cucumber (23). In pea seeds and seedlings, lipoxygenase was not compartmentalized in any particulate fraction; in pea root, lipoxygenase was localized in the lysosomal fractions (24). Grossman *et al.* (25) detected the greatest lipoxygenase activity in alfalfa in the mitochondria, followed by activity in the chloroplasts, and then in the microsomes.

From the findings of subcellular distribution of lipoxygenase by direct labeling with immunogold complexes in soybean cotyledons, it is likely that soybean lipoxygenase activity, present in the supernatant of density gradient fractions, originates from the cytoplasm, not from lysed cytoplasmic organelles (4). The sensitivity and effectiveness of the immunoelectron microscopic method facilitates determining the exact location of lipoxygenase in germinating soybean seeds and seedlings, eliminating the need for inference associated with other physicochemical methods of localization.

Success with the immunogold technique depends on maintenance of protein antigenicity and the preservation of ultrastructure. Lowicryl K 4M was developed to maximize tissue preservation and antigenicity retention through low temperature processing of the tissue. Successful usage of Lowicryl K 4M in immunocytochemical studies has been reported (13,26), even though ultrastructure embedded in this resin was inferior to that obtained with the conventional epoxy resin. Furthermore, the direct immunogold labeling method, introduced by DeMey *et al.* (27) using anti-goat immunoglobulin, is a simple and sensitive method for immunocytochemistry when stable immunogold complexes are prepared. A similar cytochemical technique has been used to localize chymotrypsin inhibitor-2 in developing barley endosperm (28).

The metabolic and physiological function of lipoxygenase in soybean cotyledons is still far from clear. Lipid bodies were hypothesized for the lipoxygenase location,

because the enzyme and its substrate usually are closely associated with each other. From the observations of its distribution in cytoplasm of all cotyledon cells, it is proposed that the function of lipoxygenase might lie in the dioxygenation of fatty acids to make their transport from lipid bodies to glyoxysomes possible (4).

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