Plant Physiol. (1991) 95, 1214–1218
0032-0889/91/95/1214/05/$01.00/0

Received for publication October 2, 1990
Accepted October 3, 1990

Soybean Leaves Contain Multiple Lipoxygenases[1]

W. Scott Grayburn, G. Russell Schneider, Thomas R. Hamilton-Kemp, Gerhard Bookjans, Kadum Ali, and David F. Hildebrand*

Departments of Agronomy and Horticulture, University of Kentucky, Lexington, Kentucky 40546

ABSTRACT

Chromatofocusing of soybean (*Glycine max* L.) leaf lipoxygenases revealed three distinct peaks of activity. Based on their isoelectric points (pIs), pH optima, and mutant analysis it appears that the leaf isozymes are different from those described from mature soybean seed. At least one leaf lipoxygenase appears to differ from those found in hypocotyls. The pIs of the main bands of the three leaf lipoxygenase peaks are 6.67, 5.91, and 5.67. The pH optima curves of three active fractions exhibit peaks at pH 6.2, 5.5, and 8.5, respectively. One of the fractions has two polypeptides with slightly different molecular weights, both of which react to soybean seed lipoxygenase antibodies. The other two fractions contain a polypeptide of unit molecular weight reacting with the lipoxygenase antibodies.

LOXs[2] (linoleate: oxygen oxidoreductase, EC 1.13.11.12) are found throughout the plant (8) and animal kingdoms (9). They catalyze, in plants, the hydroperoxidation of *cis,cis*-1,4-pentadiene structures present in unsaturated fatty acids. The physiological role of LOX in plants remains ambiguous, although roles in growth and development, senescence or wound response, and pest resistance have been suggested (13, 15, 19).

While soybean seed LOX has been extensively studied with three different isozymes described (2, 12), the diversity and characteristics of lipoxygenase(s) in soybean leaves has not been examined. In a survey of LOX activity in leaves of 28 plant species, Sekiya *et al.* (18) found soybean leaves to be among the highest in terms of activity. Soybean roots and hypocotyls have recently been found to possess LOX isozymes distinct from those found in seeds (17). This study was undertaken to examine lipoxygenase(s) in soybean leaves and to compare them to the previously characterized seed and hypocotyl isozymes.

MATERIALS AND METHODS

Preparation of Crude Plant Extracts

Leaves were obtained from soybean (*Glycine max* L.) plants grown during the spring in a greenhouse with unsupplemented sunlight. Leaves were measured at the maximum width, weighed, and ground in one-half volume of water with a mortar and pestle. Samples were centrifuged at 12,000*g* for 15 min and the supernatants were analyzed for LOX activity (11).

Enzyme Purification

The middle leaflets of soybean (*Glycine max* L. Merr.) trifoliates were harvested at random from plants growing in a greenhouse, frozen in liquid nitrogen, and ground with a mortar and pestle. Following grinding, 5 mM Na acetate (pH 4.5) was added (80 mL per 20 leaflets) and the resulting slurry was centrifuged for 7.5 min at 10,000*g*. The supernatant was made 10% in sucrose and loaded onto a Sephadex G-50 (coarse, 55 × 5 cm) (Pharmacia) column and eluted in 5 mM Na acetate, pH 4.5 at about 8 mL min^{-1}. The protein containing fractions were analyzed for LOX activity by measuring the change in absorbance at 234 nm with time (11). Sodium linoleate was used as the LOX substrate. This was adjusted to an absorbance of 0.45 with previously prepared (partially oxidized) substrate. The most active fractions were pooled and centrifuged for 1.5 h at 140,000*g* to remove microsomal particles. The resulting supernatant was loaded onto a PBE94 chromatofocusing column (Pharmacia) previously equilibrated to pH 7.4 with 25 mM imidazole HCl solution. The proteins were eluted using Polybuffer 74 (Pharmacia), which had been diluted 1 to 8 and adjusted to pH 4.0 with 1 M HCl.

Every other fraction collected from the chromatofocusing column was analyzed for LOX activity, and the approximate pIs of the LOX proteins were determined by comparison to pI standards (Bio-Rad) on IEF-PAGE gels (see below). The samples with the highest LOX activity in a single peak were pooled and concentrated by dialysis against 50% glycerol. Protein was determined by the modified Lowry method of Bensadoun and Weinstein (3).

Preparation of Leaf Antibodies

Chromatofocusing fractions with pIs centered at 6.67 and 5.67 were further purified by isoelectric focusing as described in the next section. The band from each fraction with highest LOX activity was excised and used to inoculate rabbits for antibody production.

Electrophoresis

SDS-PAGE was performed according to Laemmli (14) using 8.3% (acrylamide) running and 4% stacking gels. Native

[1] The investigation reported in this paper (No. 89-10-41) is in connection with a project of the Kentucky Agricultural Experiment Station and is published with approval of the Director.

[2] Abbreviations: LOX, lipoxygenase; pI, isoelectric point; IEF, isoelectric focusing.

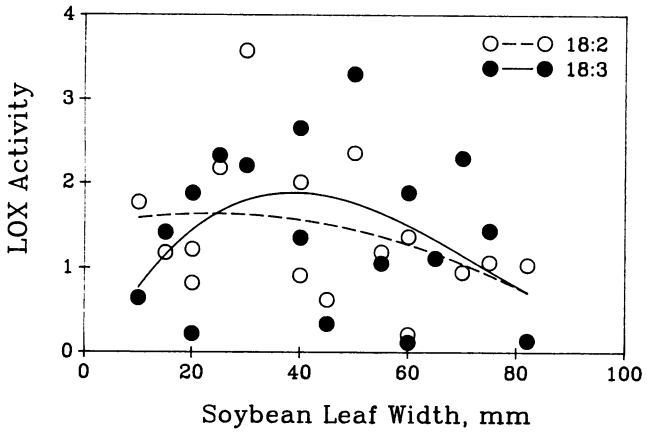

Figure 1. LOX activity of soybean leaves. Activities were determined using linoleic (18:2) or linolenic (18:3) acids as substrates at pH 6.8. Activity is expressed as μkatals/mg soluble protein.

IEF was done in a 6% polyacrylamide gel using an LKB flat bed system as described by Funk *et al.* (7). Polypeptides were visualized with Coomassie blue staining and Western immunoblotting. Western blots of both the SDS and IEF gels were performed as described by Wang and Hildebrand (20) using antibodies prepared to soybean seed LOX isozyme 1 or a mixture of antibodies to soybean leaf LOXs. The seed extract loaded on the gels was made from mature soybean cv "Century" seeds homogenized with 20 volume/weight water in a mortar and pestle. This was centrifuged at 12,000*g* for 30 min and the supernatant was used for loading.

Determination of pH Optima

The pH optima curves for active fractions from chromatofocusing were compared to soybean seed LOX 2 extracted from mature seeds of a LOX 1, LOX 3 mutant (4) using 5 mM Na acetate (pH 4.5). They were determined using four different buffer systems: 20 mM sodium acetate (pH 4–5.5), 20 mM sodium citrate (pH 5.5–7), 20 mM sodium phosphate (pH 7–8.5), and 20 mM sodium borate (pH 8.5–10). Since preliminary experiments showed a significant salt effect on the activity of leaf LOXs, the conductivity (as measured by resistance) was adjusted to a constant value with 1 M NaCl for all buffers used. The substrate used was prepared from commercially available sodium linoleate (Sigma). All other chemicals were obtained from Sigma Chemical Co.

Mutant Analysis

For studies of LOX mutants, near-isogenic lines in the 'Century' background were used (4). Leaf analyses were performed with leaves 25 to 35 mm wide from plants with half-filled pods and extracted as described above. Hypocotyls were from seeds grown for 4 d in germination towels in darkness. After homogenization in an equal volume/weight of water, hypocotyl extracts were centrifuged as described above. Mature seeds were ground in 40 volumes of water and centrifuged as described above.

RESULTS

Total Leaf LOX Activity

The relationship between leaf size and total LOX activity was investigated with two LOX substrates (Fig. 1). Leaves of approximately 30 to 40 mm had the greatest LOX activity with either linoleic or linolenic acid as substrate. For this reason, further fractionation studies were carried out with leaves 30 to 40 mm wide. Considerable variability was seen in this study. Curves that best fit the data shown in Figure 1 were generated using Sigmaplot version 3.10 (Jandel Scientific). Similar results with less variability were seen in an earlier study using leaves from an individual soybean plant, with linoleic acid as the substrate (12).

Fractionation of Leaf LOXs

Following G-50 sieving, one peak of LOX activity was seen prior to chromatofocusing. Analysis for LOX activity of fractions from the chromatofocusing column revealed three separate peaks which eluted at pH 6.3, 5.1, and 4.7. The pHs of the fractions correspond to the approximate pIs of the eluted isozymes which will be referred to as peaks 1, 2, and 3, respectively (Fig. 2). Based on activity measurements at pH 6.8, peak 1 comprised about 95%, peak 2 about 1%, and peak 3 about 4% of LOX activity in leaves.

IEF Points

A Western blot of a native IEF gel containing the peak 1, 2, and 3 fractions and an aliquot of seed extract is shown in Figure 3. All three peaks showed multiple bands immunodecorated with LOX 1 antibodies. It is unlikely that the multiple bands seen in Figure 3 are the result of proteolytic degradation. After samples had been fractionated on IEF gels (Fig. 3), high molecular mass lipoxygenase bands (at least 87 kD) were detected on SDS gels (Fig. 4). The same extracts (stored

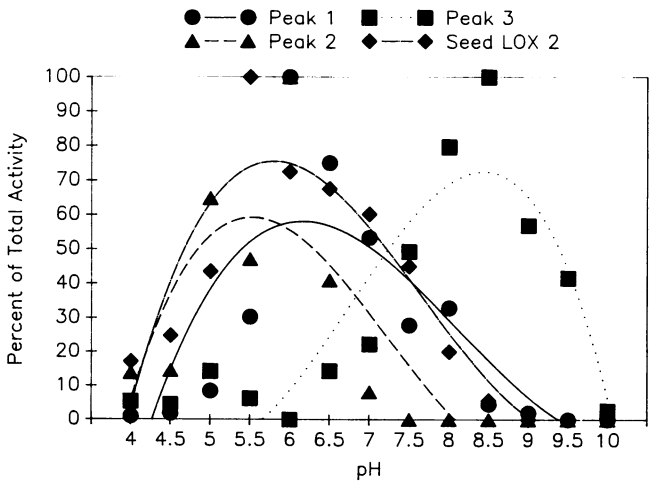

Figure 2. LOX activity of the three chromatofocusing peaks from soybean leaves compared to soybean seed LOX 2. The activities of all four LOX preparations are normalized to 100% to facilitate comparisons.

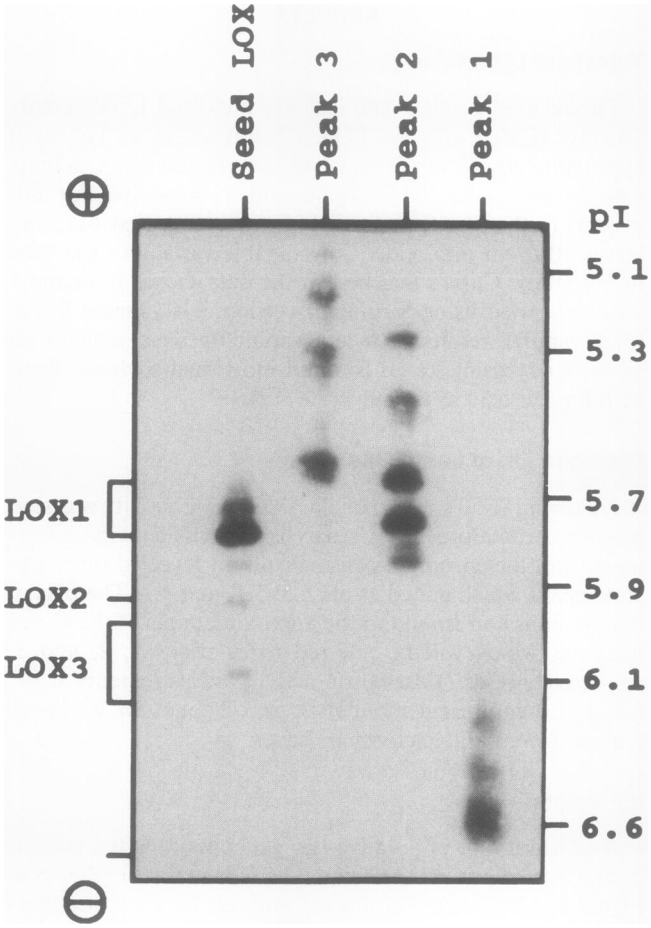

Figure 3. Western immunoblot of IEF-PAGE gel immunodecorated with soybean seed LOX 1 antibodies. Extract from mature seed of the commercial soybean cultivar 'Century' showing seed LOX 1, 2, and 3 (seed LOX). The locations of these isozymes are indicated. Soybean leaf chromatofocusing peaks 1, 2, and 3 are also shown.

frozen) were used in both cases. The range of pIs of bands seen in peak 1 were 6.1 to 6.6, in peak 2, 5.3 to 5.9, and in peak 3, 5.1 to 5.9. As can be seen in Figure 5, *Glycine max* mutants that are null for LOX 2 (−L2, −L2−L3) exhibit a slightly altered pI for LOX 1 as noted by Park and Polacco (17).

Molecular Masses

Peaks 1, 2, and 3 were run on 8.3% SDS-PAGE gels and visualized by either Coomassie blue staining (Fig. 4a) or Western blotting (Fig. 4b). The Coomassie blue-stained SDS gel shows that all LOX peaks had been purified to a great extent, although not to homogeneity (lanes 1–3 in Fig. 4a). Differences in the molecular masses of the LOX polypeptides isolated from leaves were apparent from the Western blot (Fig. 4b). The peak 1 fraction apparently contained two LOXs of slightly different molecular masses (about 93 and 87 kD), whereas the polypeptides present in the fractions containing peak 2 and peak 3 had an intermediate molecular mass of about 89 kD.

pH Optima

pH optima curves revealed that the enzymatic activities of the three peaks of activity and of LOX 2 were affected by not only pH but also by the buffer systems used (Table I). Activity of peak 1 was reduced about 70% in the citrate buffer compared to the acetate buffer. Similarly, the activities of peaks 2 and 3 and of LOX 2 from seed were lower in citrate buffer than in acetate buffer at the same pH (Table I). When the activity in citrate buffer at pH 7 was compared to that in phosphate buffer at pH 7, differences were again seen; however, all of the fractions were not affected in the same fashion. Activity of the peak 1 fraction was approximately the same in citrate and phosphate at pH 7, while the activity of peak 3 was reduced by about 50% in phosphate. The leaf lipoxygenases had similar activity in citrate and phosphate buffers, while the soybean seed LOX 2 activity was greater in phosphate than in citrate buffer (Table I). Therefore, the pH curves were normalized to the activity present in citrate buffer at or below pH 7.0. Above pH 7.0, the pH curves were normalized to the activity present in phosphate buffer. The activity displayed in Figure 2 at pH 8.5 and above is in borate buffer. Soybean seed LOX 2 has a pH optimum at approximately pH 5.8 under these conditions.

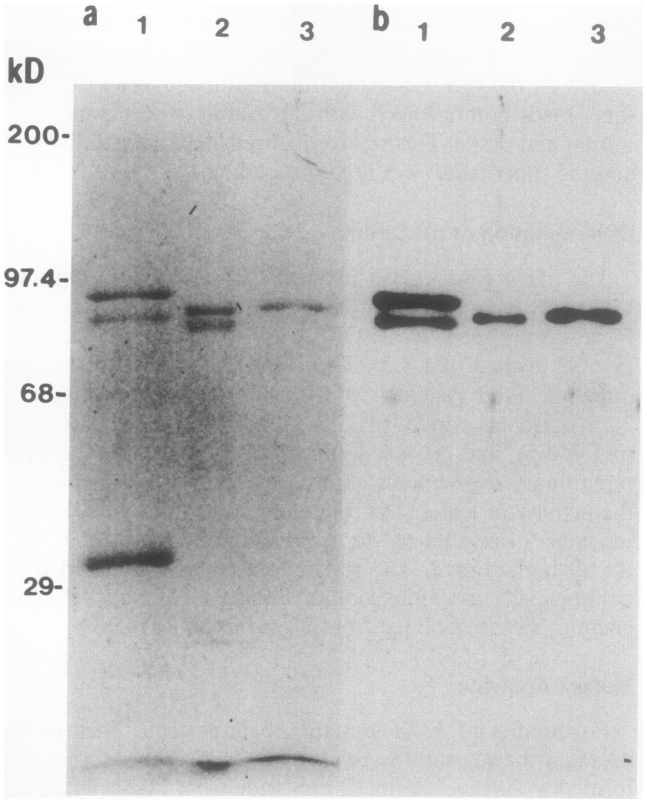

Figure 4. a, SDS-PAGE gel of soybean leaf lipoxygenase (LOX) chromatofocusing peaks 1 (lane 1), 2 (lane 2), and 3 (lane 3) stained with Coomassie blue; b, a Western immunoblot of a duplicate gel immunodecorated with soybean seed LOX 1 antibodies.

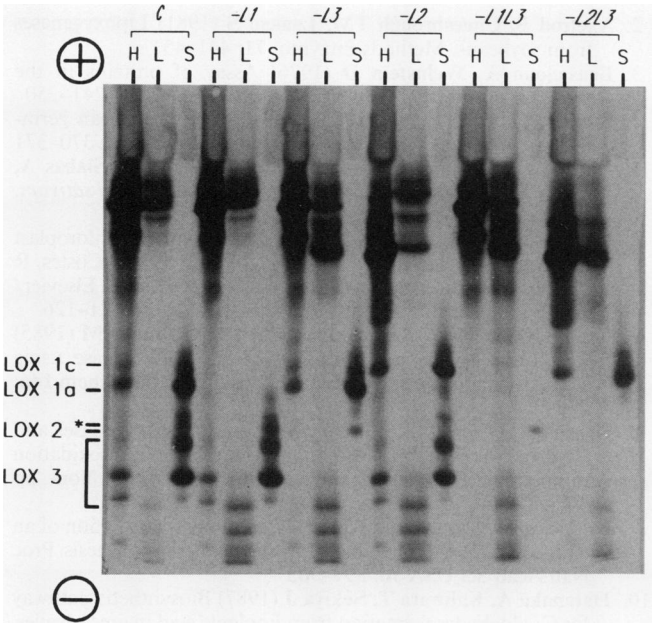

Figure 5. Western immunoblot of IEF-PAGE gel immunodecorated with soybean leaf LOX antibodies (see text). Undiluted extracts from leaves (L) and hypocotyls (H) were loaded while a 6:100 dilution of seed extracts (S) were used. The positions of seed LOX 1, 2, and 3 are indicated. C is the cultivar 'Century', which contains LOX 1 to 3. Mutants lacking one or two seed LOX isozymes are indicated. An apparent isoform of LOX 3 is shown by the asterisk (*).

DISCUSSION

The evidence obtained from both SDS and IEF gel electrophoresis suggests that all three chromatofocusing peaks are composed of several LOX proteins. The peak 1 fraction contains two polypeptides reacting with seed LOX 1 antibodies that are distinct in size (about 93 and 87 kD). Peak 1 also contains at least four isozymes and/or isoforms of LOX. Peak 2 also appears to consist of multiple polypeptides with the same molecular mass (about 89 kD), each of which has a slightly different pI ranging from about 5.3 to 5.9. Peak 3 likewise contains multiple polypeptides of the same apparent molecular mass (about 89 kD) with similar pIs ranging from about 5.1 to 5.7. The multiple pIs seen for all three peaks may represent changes in conformation of the isozyme(s) present in the peaks due to the pressure of the electromotive force used during the IEF and/or changes in conformation that are an artifact of isolation. Soybean seed LOX 3 also yields three isoforms on IEF (Figs. 3 and 5; our unpublished data; and M. Funk, personal communication). A possible fourth isoform of LOX 3 is indicated in Figure 5 by an asterisk (*). It is not known whether these multiple isoforms exist *in vivo*. The molecular mass differences for the LOXs present in peak 1 could be due to differently processed forms of the same polypeptide gene product or distinct gene products.

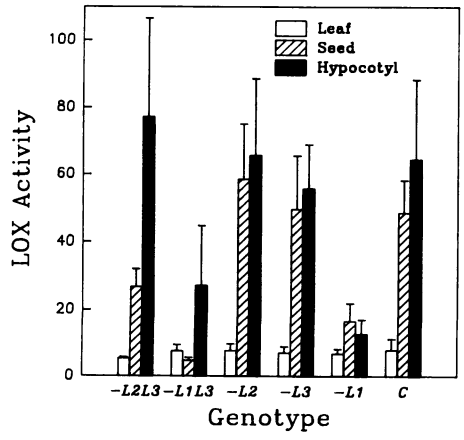

Figure 6. LOX activity of soybean leaves, hypocotyls, and seeds. *G. max* 'Century' (C) and derivatives lacking one or more seed LOX are compared. Activities were determined using linoleic acid as substrate at pH 6.8. Activity is expressed as μkatals/mg soluble protein.

Table I. *Comparison of the Activity of Various LOX Isozymes at the Same pH Using Different Buffer Systems*

pH	Buffer	Isozyme Activity			
		Leaf LOX 1	Leaf LOX 2	Leaf LOX 3	Seed LOX 2
		μkatals min^{-1} mg soluble protein^{-1}			
5.5	Acetate	30.2	2.8	0.9	61.4
	Citrate	9.5	2.1	0.0	43.0
7.0	Citrate	16.7	0.0	1.0	26.3
	Phosphate	16.2	0.0	0.5	29.0
8.5	Phosphate	1.4	0.0	2.1	2.6
	Borate	0.9	0.0	15.8	0.0

The relative abundance of activity in peak 1 appears to be related to the method of extraction. After extraction of leaves with water and 2% polyvinyl polypyrrolidone, peak 3 had more activity than peaks 1 and 2 (data not shown).

The soybean leaf LOX isozymes appear to be different from those found in soybean seeds although the molecular masses are very similar. The apparent pI range of peak 3 (5.1–5.7) is more acidic than the reported pIs of any of the seed LOX isozymes. While the pH optimum at 8.5 of peak 3 (Fig. 2) is similar to that of soybean seed LOX 1, it is nevertheless lower than the reported optimum of pH 9 for seed LOX 1 (2). The major forms of LOX in peak 1 have pIs more basic than any of the seed LOX isozymes. For soybean seed LOX 3 the reported pH optimum is very broad and is centered around pH 7, while the reported pH optimum of seed LOX 2 is 6.8. Although the pH optimum of crude seed LOX 2 obtained in our experiments was 5.8 (Fig. 2), it appears that all of the isozymes isolated from leaves are different from the LOXs isolated from seeds.

Direct evidence that leaf LOX isozymes differ from those found in seeds is shown in Figures 5 and 6. Mutations for LOX 1 to 3 affect leaf LOX isozymes differently than seed isozymes. Similar LOX activity was seen for leaves from these mutants (Fig. 6). However, hypocotyl and seed LOX activity varied in the different mutant backgrounds. Mutants lacking 1 or 2 seed LOX isozymes still show proteins that react with

leaf LOX antibodies (Fig. 5). In addition, leaf and seed LOXs have different pIs in wild-type and mutant backgrounds. Northern analysis suggests that mRNA for the three seed LOX isozymes is abundant only in embryos (1).

Soybean leaves and hypocotyls appear to possess some isozymes in common (Fig. 5). Several of the most acidic bands (peaks 2 and 3) are seen in both tissues, while the most basic bands (peak 1) are detected only in leaf extracts. Our results support the conclusion of Park and Polacco (17) that soybean seeds and hypocotyls possess distinct LOX isozymes. LOXs from pea also appear to vary in different tissues (5). The main stem and root polypeptides differed in size from the seed polypeptides. Western blot analysis identified a minimum of seven different polypeptides in peas.

The LOXs of the peak fractions 1 and 2 have low pH optima and, therefore, are candidates to be localized in the vacuole, where the pH is in the range of 5 to 6. Peak 3, on the other hand, with its high pH optimum at 8.5, would be a likely candidate to function in the stroma of the chloroplast, especially in the light. Douillard (6) concluded that LOX activity of young wheat leaves was localized in chloroplasts. Hatanaka *et al.* (10) also found LOX activity in chloroplasts in about 40 plant species. The pH optima of fractions 1 and 2 are more similar to those reported for other LOXs (16).

We have found at least three isozymes of LOX in soybean leaves which appear to be different from any of the three previously reported soybean seed isozymes. At least one of these leaf isozymes appears to be different from the previously reported hypocotyl/radical isozymes. The differential reactions of the three isozyme fractions in various buffer systems may prove to be a useful tool in determining which of the isozymes is functioning under different conditions.

ACKNOWLEDGMENTS

We thank Gary Veach, Robert T. Versluys, Udaya Chand, and Cindy Legg for technical assistance, Dr. Paul Weston for useful editorial suggestions, and Brenda Herrington for typing. This manuscript is dedicated to the memory of Dr. G. Russell Schneider whose untimely death on November 5, 1989, at the age of 36 cut short a promising career in plant physiology. Russell Schneider received his Ph.D. in plant physiology in 1987 at the University of Kentucky and joined the U.S. Department of Agriculture Southern Regional Laboratory in New Orleans, LA, in 1988.

LITERATURE CITED

1. **Altschuler M, Grayburn WS, Collins GB, Hildebrand DF** (1989) Developmental expression of lipoxygenases in soybeans. Plant Sci **63:** 151–158
2. **Axelrod B, Cheesbrough TM, Laakso S** (1981) Lipoxygenases from soybeans. Methods Enzymol **71:** 441–451
3. **Bensadoun A, Weinstein D** (1976) Assay of proteins in the presence of interfering materials. Anal Biochem **70:** 241–250
4. **Davies CS, Nielsen NC** (1987) Registration of soybean germplasm that lacks lipoxygenase isozymes. Crop Sci **27:** 370–371
5. **Domoney C, Firmin JL, Sidebottom C, Ealing PM, Slabas A, Casey R** (1990) Lipoxygenase heterogeneity in *Pisum sativum*. Planta **181:** 35–43
6. **Douillard R** (1980) Characterization and meaning of chloroplast lipoxygenase activities. *In* P Mazliak, P Benviste, C Costes, R Douce, eds, Biogenesis and Function of Plant Lipids. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 121–126
7. **Funk MO, Whitney MA, Hausknecht EC, O'Brian EM** (1985) Resolution of the isozymes of soybean lipoxygenase using isoelectric focusing and chromatofocusing. Anal Biochem **146:** 246–251
8. **Gardner HW** (1980) Lipid enzymes: lipases, lipoxygenases and "hydroperoxidases". *In* MG Simic, M Karel, eds, Autoxidation in Food and Biological Systems. Plenum Press, New York, pp 447–504
9. **Hamberg M, Samuelsson B** (1973) Detection and isolation of an endoperoxide, intermediate in prostaglandin biosynthesis. Proc Natl Acad Sci USA **70:** 899–903
10. **Hatanaka A, Kajiwara T, Sekiya J** (1987) Biosynthetic pathway for C_6-aldehydes formation from linolenic acid in green leaves. Chem Phys Lipids **44:** 341–361
11. **Hildebrand DF, Hymowitz T** (1981) Two soybean genotypes lacking lipoxygenase-1. J Am Oil Chem Soc **58:** 583–586
12. **Hildebrand DF, Hamilton-Kemp TR, Legg CS, Bookjans G** (1988) Plant lipoxygenases: occurrence, properties and possible functions. *In* DD Randall, DG Blevins, WH Campbell, eds, Current Topics in Plant Biochemistry and Physiology Vol 7. University of Missouri-Columbia, Interdisciplinary Plant Biochemistry & Physiology Group, Columbia, MO, pp 201–219
13. **Hildebrand DF** (1989) Lipoxygenases. Physiol Plant **76:** 249–253
14. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
15. **Mack AJ, Peterman TK, Siedow JN** (1987) Lipoxygenase isozymes in higher plants biochemical properties and physiological role. Curr Top Biol Med Res **13:** 127–154
16. **Mulliez E, Leblanc J-P, Girerd J-J, Rigaud M, Chottard JC** (1987) 5-Lipoxygenase from potato tubers. Improved purification and physicochemical characteristics. Biochim Biophys Acta **916:** 13–23
17. **Park TK, Polacco JC** (1989) Distinct lipoxygenase species appear in the hypocotyl/radicle of germinating soybean. Plant Physiol **90:** 285–290
18. **Sekiya J, Kajiwara T, Munechika K, Hatanaka A** (1983) Distribution of lipoxygenase and hydroperoxide lyase in the leaves of various plant species. Phytochemistry **22:** 1867–1869
19. **Vick BA, Zimmerman DC** (1987) Oxidative systems for modification of fatty acids: the lipoxygenase pathway. *In* PK Stumpf, ed, The Biochemistry of Plants, Vol 9. Academic Press, Orlando, FL, pp 53–90
20. **Wang XM, Hildebrand DF** (1987) Effect of a substituted pyridazinone on the decrease of lipoxygenase activity in soybean cotyledons. Plant Sci **51:** 29–36