

# Lipoxygenase, Hydroperoxide Isomerase, and Hydroperoxide Cyclase in Young Cotton Seedlings<sup>1</sup>

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## ABSTRACT

Lipoxygenase was demonstrated in young cotton seedlings. It catalyzed the oxygenation of linoleic or linolenic acid, predominantly at carbon 13, and its molecular weight was estimated by gel filtration to be 100,000. Hydroperoxide isomerase was also present and converted hydroperoxylinoleic or hydroperoxylinolenic acid to  $\alpha$ - or  $\gamma$ -ketols. The enzyme utilized the 13-hydroperoxy isomer in preference to the 9 isomer and its molecular weight was estimated at 250,000 by gel filtration. In addition, hydroperoxide cyclase, which catalyzes the conversion of 13-hydroperoxylinolenic acid to 12-oxo-phytodienoic acid, was present. Hydroperoxide isomerase and hydroperoxide cyclase activities could not be separated by gel filtration and ion-exchange chromatography experiments, indicating the two enzyme activities may be associated with the same protein. The activities of all three enzymes were very low in the seed but increased immediately after germination, reached a maximum after 3 to 4 days, and then declined. The results suggest a role, as yet unknown, for these enzymes during early plant development.

Lipoxygenase, hydroperoxide isomerase, and hydroperoxide cyclase are enzymes which transform certain polyunsaturated fatty acids to oxygenated metabolites (Fig. 1). Lipoxygenase (EC 1.13.11.12) catalyzes the oxygenation of fatty acids containing a *cis*-1,*cis*-4-pentadiene structure to fatty acid hydroperoxides. The enzyme is present in a wide range of plant tissues (1) and, in the last few years, has been demonstrated in some animal tissues (2, 9, 10, 16, 19). Plant lipoxygenase is active with a number of polyunsaturated fatty acids in the 18- to 22-carbon range (13), but the natural substrate for the plant enzyme is probably linoleic or linolenic acid, the two most abundant polyunsaturated fatty acids in plant tissues. *In vitro* experiments have shown that these two acids are converted to either *n*-6 or *n*-10 hydroperoxides, depending upon the enzyme source, isoenzyme composition, pH, or O<sub>2</sub> concentration (3). In animal tissues, lipoxygenase can catalyze the oxygenation of linoleic acid to the *n*-6 or *n*-10 hydroperoxides as in rat testes (19), or the oxygenation of arachidonic acid to the *n*-9 hydroperoxide as in human (10) or bovine (16) blood platelets. Lipoxygenase from rabbit leukocytes oxygenates the *n*-16 carbon of arachidonic acid (2).

Hydroperoxide isomerase catalyzes the conversion of fatty acid hydroperoxides to  $\alpha$ - or  $\gamma$ -ketol compounds. The enzyme was first reported in flaxseed by Zimmerman (27) and was later demonstrated in several other plants (4, 6, 23, 26). Grossman *et al.* (8) have recently reported evidence for hydroperoxide isomerase in a

microsomal preparation from rat testes.

Hydroperoxide cyclase was first characterized by Zimmerman and Feng (28) as an enzyme which catalyzes the cyclization of 13-hydroperoxylinolenic acid to an 18-carbon fatty acid containing a cyclopentenone ring. The product, 8-[2-(*cis*-2'-pentenyl)-3-oxo-*cis*-4-cyclopentenyl]octanoic acid, was given the common name 12-oxo-phytodienoic acid. Other fatty acid hydroperoxides can serve as substrates, but studies by us (24) showed that the hydroperoxide must possess *n*-3 unsaturation to be a substrate for the cyclase enzyme. We have demonstrated the presence of the enzyme in a wide variety of plant tissues (23).

The role of the products of these enzymes in plant metabolism is not known. In animal metabolism, however, *in vitro* experiments have shown that fatty acid hydroperoxides can accelerate the cyclooxygenase reaction, which is the initial step in the conversion of 20-carbon, polyunsaturated fatty acids to prostaglandins, thromboxanes, and prostacyclin (12). Activation of splenic cell guanylate cyclase by fatty acid hydroperoxides has also been demonstrated (7). Turner *et al.* (21) have provided evidence that 12-hydroxy-5,8,10,14-eicosatetraenoic acid, formed by enzymic reduction of the 12-hydroperoxy group, is a mediator of neutrophilic leukocyte chemotaxis. Whether these effects have *in vivo* significance has not been established. For  $\alpha$ - or  $\gamma$ -ketols of fatty acids and for 12-oxo-PDA<sup>2</sup>, no physiological role in either plants or animals has yet been demonstrated. Here, we report on the presence and properties of lipoxygenase, hydroperoxide isomerase, and hydroperoxide cyclase in young cotton seedlings.

## MATERIALS AND METHODS

**Chemicals.** Linoleic and linolenic acids were obtained from Nu Chek Prep, Inc.<sup>3</sup>, Elysian, MN. Platinum oxide (Adam's catalyst) was purchased from Matheson, Coleman, and Bell, Norwood, OH, and methoxyamine-HCl in pyridine (MOX reagent) and *N,O*-bis(trimethylsilyl)-trifluoroacetamide were purchased from Pierce Chemical Co., Rockford, IL. Gas-Chrom Q (100/120 mesh) and the silicone phase DC LSX-3-0295 for GC were obtained from Applied Sciences, Inc., State College, PA. All TLC separations were accomplished with Anasil HF precoated TLC plates obtained from Analabs, Inc., New Haven, CT.

**Growth Conditions.** Delinted cotton seeds (*Gossypium hirsutum* L. var. Stoneville 213) were soaked overnight in H<sub>2</sub>O without prior sterilization and then planted between two sheets of moist paper toweling 2 cm from the top. The moist towels were placed

<sup>2</sup> Abbreviations: 12-oxo-PDA, 12-oxo-phytodienoic acid; TMS, trimethylsilyloxy.

<sup>3</sup> Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

<sup>1</sup> This work conducted in cooperation with the North Dakota Agricultural Experiment Station, Paper No. 1061.

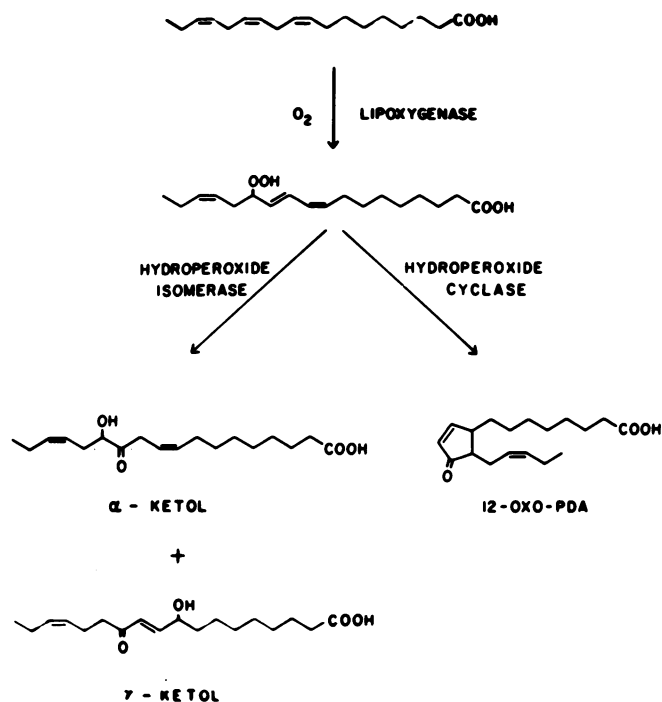


FIG. 1. Reactions catalyzed by lipoxygenase, hydroperoxide isomerase, and hydroperoxide cyclase with linolenic acid as substrate.

on a sheet of waxed paper, rolled up, and placed upright in a beaker of H<sub>2</sub>O. The seeds were germinated in the dark at 27 C, and the time of planting was considered to be time zero in developmental studies. Seedling length (hypocotyl plus root) was used as a guide to the physiological age of the seedling: 1 day, 2.5 cm; 2 days, 6 to 7 cm; 3 days, 10 to 13 cm; 4 days, 15 to 19 cm; 5 days, 19 to 25 cm; 6 days, 25 to 32 cm.

**Enzyme Extraction.** Whole seedlings with testae removed were weighed, transferred to a mortar, frozen in liquid N<sub>2</sub>, and ground to a powder with a pestle. Enzymes were extracted by grinding with 50 mM K-phosphate buffer (pH 6.0) containing 0.1% Triton X-100 (v/v). When the enzyme extract was to be applied to an ion-exchange chromatography column, the extraction buffer was 50 mM imidazole (pH 6.0) containing 0.1% Triton X-100 (v/v). For each g fresh weight of seedlings, 3 ml buffer was used. The homogenate was filtered through cheesecloth and centrifuged for 10 min at 12,000g at 5 C. The lipid material that floated to the top during centrifugation was removed and the supernatant was decanted and used as the enzyme source. The presence of Triton X-100 in the extraction buffer increased the activity of all three enzymes approximately 2-fold and was necessary to preserve enzyme activity during storage. Hydroperoxide isomerase and hydroperoxide cyclase activities were lost rapidly if the extracts were frozen, but activities could be retained for 3 to 4 days if kept at 4 C.

**Determination of Lipoxygenase Positional Specificity.** To determine the 13:9 positional specificity of oxygenation by cotton lipoxygenase, linoleic and linolenic acids were converted to hydroperoxides by reacting them with lipoxygenase partially purified by gel filtration (Fig. 4A). Each reaction mixture contained 2.9 ml 50 mM K-phosphate buffer (pH 6.0), 20 μl 8 mM linoleic or linolenic acid substrate solution prepared according to Surrey (20), and 0.1 ml of the partially pure lipoxygenase fraction. The reaction was allowed to proceed for 5 min at 24 C and was terminated by the addition of 6 ml dichloromethane-methanol (2:1, v/v). The pH was adjusted to 4, the dichloromethane phase was dried with anhydrous sodium sulfate, and the solvent was evaporated. The fatty acid products were esterified with diazomethane and the

double bonds were saturated by hydrogenation, which also reduced hydroperoxide groups to hydroxyl groups. TMS derivatives of the hydroxyl groups were prepared and the samples were analyzed by GC-MS. The 13:9 hydroperoxide ratio was determined by comparison of the areas of the peaks generated by selected ion monitoring of the masses *m/e* 173 plus 315 (13-isomer) and *m/e* 229 plus 259 (9-isomer).

**Determination of Hydroperoxide Isomerase Substrate Specificity.** An extract of 3-day-old etiolated cotton seedlings was reacted with a solution containing predominantly 13-hydroperoxylinolenic acid (about 74%) and with a solution of predominantly 9-hydroperoxylinolenic acid (about 83%) to determine the relative activity of hydroperoxide isomerase toward each substrate. The 13 isomer solution was prepared from soybean lipoxygenase at pH 9 (5) and the 9 isomer solution was prepared from tomato lipoxygenase at pH 5.5 (14). The concentration of hydroperoxide in each solution was adjusted to 20 μM and the pH was adjusted to 6 prior to the addition of cotton seedling extract. The enzyme rates were measured spectrophotometrically and protein measurements were made by the use of Coomassie brilliant blue G-250 dye (17).

**Isolation of Products from Reaction of Linolenic Acid with a Cotton Seedling Extract.** A typical reaction mixture contained 600 ml 50 mM K-phosphate buffer (pH 6), 30 ml 8 mM linolenic acid substrate solution, and 15 ml cotton seedling extract. After 90 min, the reaction was stopped, the pH was adjusted to 4, and the products were extracted with 500 ml chloroform-methanol (2:1, v/v). TLC separation of the products was accomplished by three developments with chloroform-acetic acid (100:1, v/v).

**Characterization of Products.** Double bonds were reduced by hydrogenation with platinum oxide catalyst; carbonyl groups were reduced to hydroxyl groups with sodium borohydride; hydroxyl groups were oxidized to carbonyl groups with chromium trioxide; and double bond positions were determined by oxidative ozonolysis. Methyloxime derivatives of carbonyls were prepared from MOX reagent and TMS derivatives of hydroxyl groups were prepared with *N,O*-bis(trimethylsilyl)-trifluoroacetamide reagent. These methods have been described in detail in a previous paper (25).

IR spectra were obtained with a Perkin-Elmer model 337 spectrophotometer. Microliquid cells with a 0.50-mm path length were used with carbon tetrachloride as solvent. UV spectra were recorded with a Beckman DK-2 spectrophotometer with ethanol solvent. Mass spectra were recorded with a Varian/MAT 112S GC-MS system; the glass column was 2 m × 2 mm i.d. containing 3% DC LSX-3-0295 on 100/120 mesh Gas-Chrom Q and was temperature-programmed from 165 to 220 C at 2 C/min. Selected ion monitoring was accomplished with a Hewlett-Packard 5992A GC-MS. The glass column, similar to the one described above, was temperature-programmed from 200 to 240 C at 2.5 C/min.

**Enzyme Purification.** For gel filtration, 3 ml of a cotton seedling extract was applied to a Sephacryl S-300 (Pharmacia, Inc.) column (2.6 × 37 cm). The protein was eluted with 50 mM K-phosphate buffer (pH 6) containing 0.1% Triton X-100 (v/v) and collected in 1.8-ml fractions. When protein fractions from gel filtration were to be purified further by ion-exchange chromatography, the eluting buffer was 50 mM imidazole (pH 6.0) containing 0.1% Triton X-100 (v/v). Mol wt was estimated by plotting *K<sub>av</sub>* versus log mol wt of proteins of known mol wt (thyroglobulin, 669,000; ferritin, 440,000; aldolase, 158,000; BSA, 67,000; and myoglobin, 16,890). The void volume was determined with blue dextran.

Ion-exchange chromatography was accomplished with a 1.6 × 36.5-cm column of DEAE-Sephadex A-50 (Pharmacia, Inc.). The eluent was a continuous gradient of increasing ionic strength, from 50 mM imidazole (pH 6.0) containing 0.1% Triton X-100 (v/v) to 50 mM imidazole (pH 6.0) containing 0.1% Triton X-100 (v/v) and 0.5 M NaCl.

Protein concentration in chromatography fractions was not

measured due to the presence of Triton X-100.

**Enzyme Assays.** Lipoxygenase was measured spectrophotometrically at 234 nm (20) or with a Clark-type polarographic electrode. The initial O<sub>2</sub> concentration was assumed to be 250 μM.

Hydroperoxide isomerase was assayed spectrophotometrically as described previously by measuring the decrease in *A* at 234 nm due to the disappearance of the conjugated fatty acid hydroperoxide (29).

Hydroperoxide cyclase was measured by GC-MS selected ion monitoring of 12-oxo-PDA, formed from 13-hydroperoxylinolenic acid. A 20-carbon analog of 12-oxo-PDA was added as an internal standard. The reaction mixture contained 2.5 ml 50 mM K-phosphate or imidazole buffer (pH 6.0), 0.5 ml of a solution of 13-hydroperoxylinolenic acid (about 240 μM) (5), 5 μg of the 20-carbon cyclic fatty acid internal standard prepared from 11, 14, 17-icosatrienoic acid (24), and 180 to 600 μl enzyme. After 2 to 5 min at 24 C, the reaction was terminated by the addition of 6 ml dichloromethane-methanol (2:1, v/v), the pH was adjusted to 4, the dichloromethane phase was dried, and the solvent was evaporated. The products were esterified with diazomethane and the double bonds were saturated by hydrogenation. The amount of 12-oxo-PDA formed was determined with GC-MS by comparing the intensities of ions *m/e* 83, 153, and 240 due to saturated 12-oxo-PDA (28) with the intensities of ions *m/e* 83, 153, and 268 due to the saturated 20-carbon cyclic internal standard (24).

## RESULTS

### LIPOXYGENASE POSITIONAL SPECIFICITY

GC-MS analysis of the hydrogenated TMS derivatives of the hydroperoxide products resulting from a reaction between linoleic acid and an extract of cotton seedlings showed that 94% of the product was the 13-hydroperoxide isomer and 6% was the 9-hydroperoxide isomer. With linolenic acid, the 13-hydroperoxide isomer accounted for 93% of the two products.

### HYDROPEROXIDE ISOMERASE SUBSTRATE SPECIFICITY

An extract of cotton seedlings was reacted with a solution containing 9-hydroperoxylinoleic acid and a solution containing 13-hydroperoxylinoleic acid, and the reaction rates were measured spectrophotometrically. The specific activity of the enzyme with the 13-hydroperoxy isomer was 885 nmol min<sup>-1</sup> mg<sup>-1</sup> and that with the 9-hydroperoxy isomer was 26 nmol min<sup>-1</sup> mg<sup>-1</sup>, a ratio of 34:1. This ratio is similar to that reported by Feng and Zimmerman (5) for hydroperoxide isomerase from flaxseed, which preferentially metabolized the 13 isomer by a ratio of 36:1. The product of the isomerase reaction with 9-hydroperoxylinoleic acid was 9-hydroxy-10-oxo-*cis*-9-octadecenoic acid, confirmed by comparison of the mass spectrum of its methyloxime, TMS, methyl ester derivative with a previously published spectrum of this compound (5).

### CHARACTERIZATION OF PRODUCTS

When linolenic acid was reacted with an extract of 4-day-old etiolated cotton seedlings, three major products were present after separation by TLC (product A, about 10%, product B, about 60% and product C, about 25% of the total products formed). The hydroperoxide products of lipoxygenase catalysis were not present, indicating that they had been completely converted to other products. TLC showed no evidence for the formation of 12-oxo-*trans*-10-dodecenoic acid, a product of 13-hydroperoxylinoleic or linolenic acid metabolism in some plants catalyzed by a hydroperoxide lyase enzyme (22). Product A (*R<sub>r</sub>*, 0.13), product B (*R<sub>r</sub>*, 0.41), and product C (*R<sub>r</sub>*, 0.46) were esterified with diazomethane and characterized as described below.

**Product A.** The IR spectrum showed absorption at 3511 cm<sup>-1</sup> due to a hydroxyl group, 3000 cm<sup>-1</sup> due to unsaturation, 1739 cm<sup>-1</sup> from the ester carbonyl, and at 1697, 1678, and 1630 cm<sup>-1</sup>, characteristic of an α,β-unsaturated carbonyl. Absorption at 972 cm<sup>-1</sup> indicated the presence of a *trans* double bond.

When the methyloxime and trimethylsilyloxy derivative was prepared for this compound and then analyzed by GC-MS, the following mass fragments were present: *m/e* 425 [*M*]<sup>+</sup>, *m/e* 394 [*M*-OCH<sub>3</sub>]<sup>+</sup>, *m/e* 304 (loss of TMSOH from *m/e* 394), *m/e* 268 [*M*-(CH<sub>2</sub>)<sub>7</sub>COOCH<sub>3</sub>]<sup>+</sup>, and *m/e* 259 [CH(OTMS)(CH<sub>2</sub>)<sub>7</sub>COOCH<sub>3</sub>]<sup>+</sup>. The mass spectrum indicated that the hydroxyl was at carbon 9, but the position of the carbonyl remained ambiguous.

Reduction of compound A with sodium borohydride and preparation of the TMS derivative resulted in a compound with mass fragments at *m/e* 387 [*M*-CH<sub>3</sub>CH<sub>2</sub>CHCH(CH<sub>2</sub>)<sub>2</sub>]<sup>+</sup> and *m/e* 313 [*M*-(CH<sub>2</sub>)<sub>2</sub>COOCH<sub>3</sub>]<sup>+</sup>, clearly indicating oxygenation at carbons 9 and 12. Therefore, the carbonyl oxygen was assigned to carbon 12. Since the IR spectrum indicated a conjugated carbonyl, this meant that the double bond at carbon 9 had been isomerized to the *trans*-10 position. There was no evidence for the isomerization of the double bond at carbon 15. Thus, compound A contained a γ-ketol functional group and was identified as the methyl ester of 9-hydroxy-12-oxo-*cis*-15,*trans*-10-octadecadienoic acid.

**Product B.** The IR spectrum of product B demonstrated absorption at 3492 cm<sup>-1</sup> due to a hydroxyl, 3014 cm<sup>-1</sup> due to unsaturation, 1741 cm<sup>-1</sup> from the ester carbonyl, and 1715 cm<sup>-1</sup> from a carbonyl. There was no absorbance in the 975 cm<sup>-1</sup> region, indicating the absence of a *trans* double bond.

The methyloxime, TMS derivative produced a mass spectrum with mass fragments at *m/e* 410 [*M*-CH<sub>3</sub>]<sup>+</sup>, *m/e* 356 [*M*-C<sub>5</sub>H<sub>9</sub>]<sup>+</sup>, *m/e* 192 [C(NOCH<sub>3</sub>)CH<sub>2</sub>CH=CH(CH<sub>2</sub>)<sub>7</sub>COOCH<sub>3</sub>-(OCH<sub>3</sub>)<sub>2</sub>]<sup>+</sup>, and *m/e* 171 [CH<sub>3</sub>CH<sub>2</sub>CH=CHCH<sub>2</sub>CH(OTMS)]<sup>+</sup>. These fragments confirmed the position of the hydroxyl at carbon 13 and the carbonyl at carbon 12. Oxidative ozonolysis produced azelaic acid, indicating that a double bond remained at carbon 9. There was no evidence for the migration of the double bond at carbon 15. Therefore, product B was identified as the methyl ester of 12-oxo-13-hydroxy-*cis*-9,*cis*-15-octadecadienoic acid.

**Product C.** The IR spectrum of this compound showed absorptions at 3002 cm<sup>-1</sup> from unsaturation, 1741 cm<sup>-1</sup> due to the ester carbonyl, and 1,713 cm<sup>-1</sup> from α,β-unsaturation of a carbonyl in a five-membered ring (15). No *trans* double bonds were present. UV absorption at 221 nm also indicated carbonyl conjugation with a double bond. The mass spectrum of product C showed a molecular ion at *m/e* 306 and fragment ions at *m/e* 275 [*M*-OCH<sub>3</sub>]<sup>+</sup>, *m/e* 238 [*M*-C<sub>5</sub>H<sub>9</sub>]<sup>+</sup>, and *m/e* 206 (loss of CH<sub>3</sub>OH from *m/e* 238). The spectrum was identical with that reported by Zimmerman and Feng (28) for the methyl ester of 12-oxo-PDA. The position of the exocyclic double bond was determined by oxidative ozonolysis of the compound after saturation of the ring double bond. This was accomplished by reduction with sodium borohydride, which reduced the keto group and the ring double bond. The resulting hydroxyl group was oxidized back to a keto group with chromium trioxide. After oxidative ozonolysis, the methyloxime derivative was prepared. Mass fragments at *m/e* 341 [*M*]<sup>+</sup>, *m/e* 310 [*M*-OCH<sub>3</sub>]<sup>+</sup>, *m/e* 184 [*M*-(CH<sub>2</sub>)<sub>7</sub>COOCH<sub>3</sub>]<sup>+</sup> (base peak), and *m/e* 152 (loss of CH<sub>3</sub>OH from *m/e* 152) indicated that the ozonolysis product was the methyloxime derivative of methyl 8-(2-carbomethoxymethyl-3-oxo-cyclopentyl)-octanoate. Characterization of this product confirmed the position of the exocyclic double bond at carbon 15. Thus, we concluded that product C was 12-oxo-PDA.

### pH OPTIMA OF COTTON LIPOXYGENASE AND HYDROPEROXIDE-METABOLIZING ENZYMES

An extract of 3-day-old whole seedlings was used to determine the pH optimum for the enzymes. Lipoxygenase, tested with either

linoleic or linolenic acid as the substrate (Fig. 2A), was most active in the pH range of 5.5 to 6.5 for both acids.

Hydroperoxide conversion was measured with the 13-hydroperoxy isomer of either linoleic or linolenic acid as substrate (Fig. 2B). For 13-hydroperoxylinoleic acid, the rates measured were due to hydroperoxide isomerase activity but, for 13-hydroperoxylinolenic acid, the rates reflected a combination of hydroperoxide isomerase and hydroperoxide cyclase activities. With linoleic acid hydroperoxide, the optimum occurred at pH 6.5 and, for linolenic acid hydroperoxide, the maximum was pH 5.

#### DEVELOPMENTAL CHANGES IN ENZYME ACTIVITY

Figure 3A shows the changes in activity of lipoxygenase and hydroperoxide isomerase in young etiolated cotton seedlings during the first few days of germination. The substrates were linoleic acid and 13-hydroperoxylinoleic acid. Lipoxygenase activity was very low in the dry seed but increased rapidly after germination and reached its maximum level between 2 and 4 days. Likewise, hydroperoxide isomerase had a low level of activity in the seed, reached a high level after 3 to 4 days, and then declined. A similar pattern was observed for the activity of both enzymes when linolenic acid and 13-hydroperoxylinolenic acid were the substrates, except that the activities were reduced (Fig. 3B). The rate of disappearance of hydroperoxide in this case was due to both hydroperoxide isomerase and hydroperoxide cyclase activities.

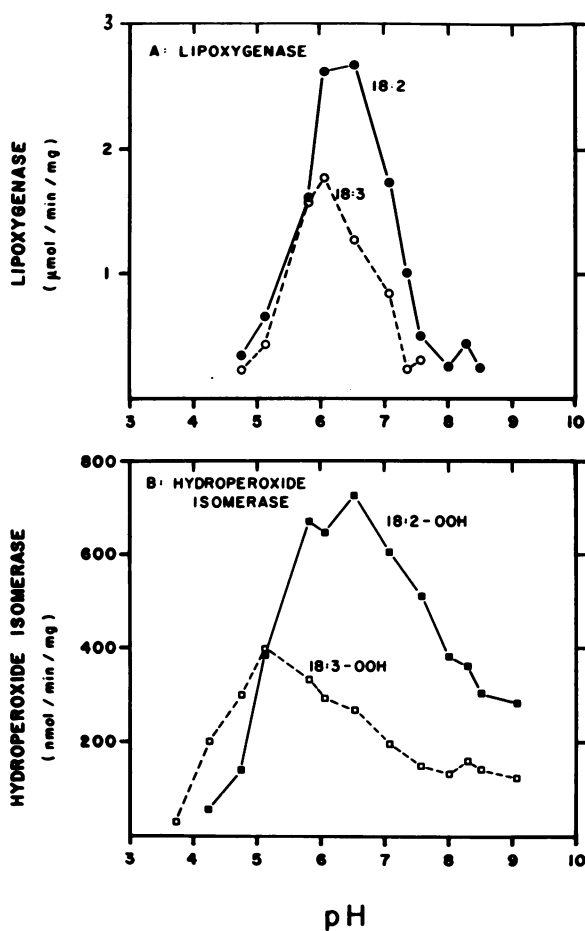


FIG. 2. Lipoxygenase (A) and hydroperoxide isomerase (B) activity from 3-day-old, dark-grown cotton seedlings as a function of pH. The substrates were: (●), linoleic acid; (○), linolenic acid; (■), 13-hydroperoxylinoleic acid; and (□), 13-hydroperoxylinolenic acid. The pH values were 3.7 to 5.1 with 50 mM citrate buffer, 5.8 to 8.3 with 100 mM K-phosphate buffer, and 8.0 to 9.1 with 50 mM Tricine buffer.

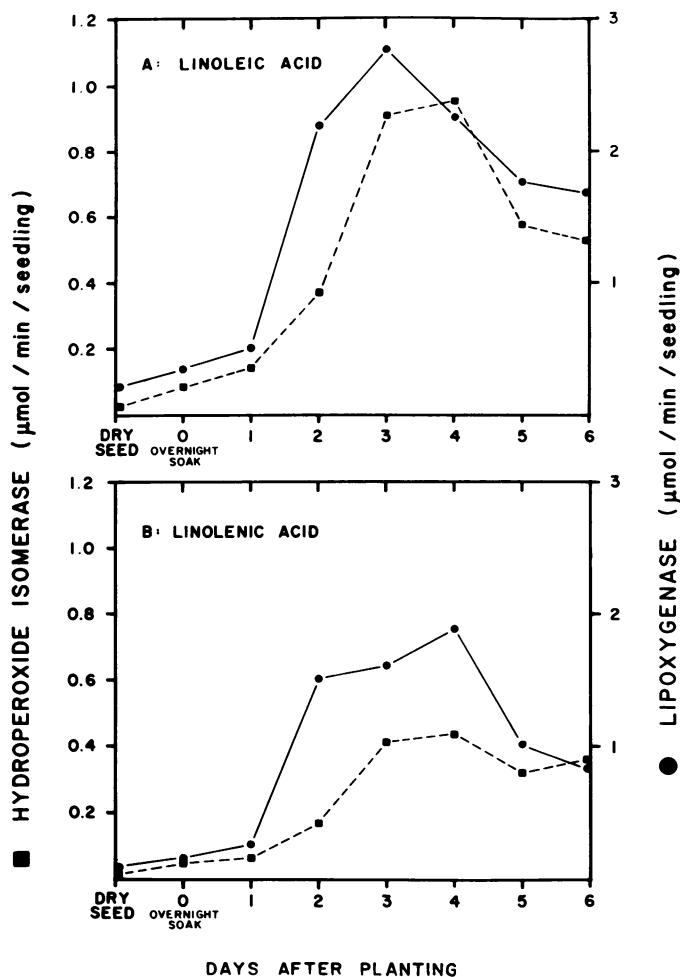


FIG. 3. Effect of age of dark-grown cotton seedlings on the activity of (●) lipoxygenase and (■) hydroperoxide isomerase. Linoleic acid (A) and linolenic acid (B) were the substrates for the lipoxygenase reaction, and the 13-hydroperoxide isomer of each was the substrate for the hydroperoxide isomerase reaction.

#### ENZYME PURIFICATION

When an extract of cotton seedlings was applied to a Sephacryl S-300 column calibrated with known proteins (Fig. 4A), the hydroperoxide isomerase and hydroperoxide cyclase eluted together (Fig. 4B). From a plot of  $K_{av}$  versus log mol wt of known proteins (Fig. 4B, inset), the mol wt for cotton hydroperoxide isomerase and cyclase was estimated at 250,000. The mol wt of cotton lipoxygenase was approximately 100,000.

A hydroperoxide isomerase-hydroperoxide cyclase preparation, partially purified by gel filtration under the conditions used in the chromatogram of Figure 4, was further purified by ion-exchange chromatography on DEAE-Sephadex A-50 (Fig. 5). Although the protein concentration of each fraction was not measured due to the presence of Triton X-100, we observed that a yellow protein present in the partially pure gel filtration preparation was separated from hydroperoxide isomerase and hydroperoxide cyclase by this procedure. The yellow protein was present in fractions 9 to 16. Hydroperoxide isomerase and hydroperoxide cyclase activities again eluted together, and this protein fraction was colorless.

#### DISCUSSION

Lipoxygenase has not previously been reported in cottonseeds, perhaps because of its very low activity. Although the seeds of many plants have active lipoxygenase synthesized during seed

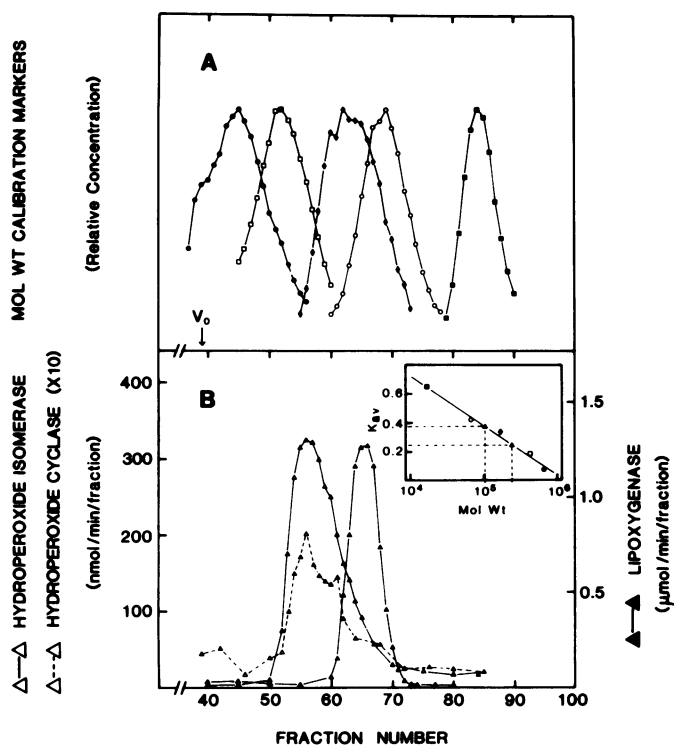


FIG. 4. Gel filtration chromatography on a Sephacryl S-300 column ( $2.6 \times 37$  cm). A, resolution of mol wt calibration proteins: thyroglobulin, 669,000; ferritin, 440,000; aldolase, 158,000; BSA, 67,000; and myoglobin, 16,890. B, separation of hydroperoxide isomerase ( $\Delta$ — $\Delta$ ), hydroperoxide cyclase ( $\Delta$ — $\Delta$ ), and lipoxygenase ( $\blacktriangle$ — $\blacktriangle$ ) from an extract of 3-day-old cotton seedlings. Inset, estimation of mol wt by a plot of  $K_{av}$  versus mol wt (log scale). The 1.8-ml fractions were eluted with 50 mM K-phosphate buffer (pH 6.0) containing 0.1% Triton X-100.

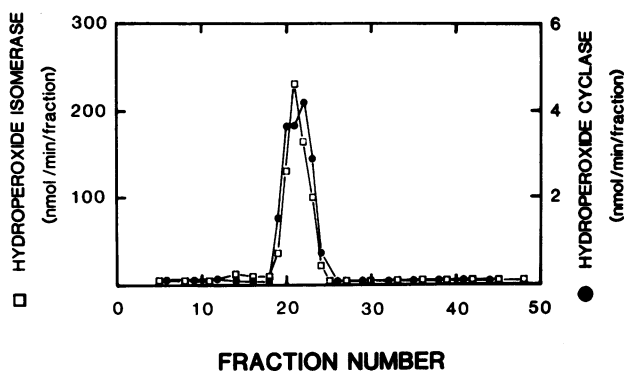


FIG. 5. Ion-exchange chromatography of a cotton extract, which had been partially purified by gel filtration (Fig. 4B), showing hydroperoxide isomerase ( $\square$ ) and hydroperoxide cyclase ( $\bullet$ ) activities. The column was DEAE-Sephadex A-50 ( $1.6 \times 36.5$  cm) with 4-ml fractions. The eluent was 50 mM imidazole buffer (pH 6.0) containing 0.1% Triton X-100 with a continuous gradient of increasing ionic strength from 0 to 0.5 M NaCl.

development, other plant seeds have very low or no lipoxygenase activity. However, many of these seeds have now been shown to synthesize lipoxygenase rapidly, shortly after germination. In addition to cotton seedling reported here, other plants with high lipoxygenase activity observed in the seedling, but not in the seed, are cucumber (18), watermelon (22), and sunflower (unpublished data). The rapid increase in lipoxygenase activity shortly after germination supports the view that the enzyme has a special function during this period.

The properties of lipoxygenase, hydroperoxide isomerase, and

hydroperoxide cyclase from cotton seedlings are remarkably similar to those enzymes from flaxseed. In both cases, the predominant product of lipoxygenase is the 13-hydroperoxide isomer of linoleic or linolenic acid. Hydroperoxide isomerase from cotton seedlings, like flaxseed, utilizes the 13-hydroperoxide isomer preferentially over the 9-hydroperoxide isomer. It catalyzes the synthesis of an  $\alpha$ -ketol (12-oxo-13-hydroxy-*cis*-9,*cis*-15-octadecadienoic acid) as the major product and a  $\gamma$ -ketol (9-hydroxy-12-oxo-*cis*-15,*trans*-10-octadecadienoic acid) as a minor product from 13-hydroperoxylinolenic acid. Hydroperoxide cyclase from both sources catalyzes the synthesis of 12-oxo-PDA, which accounts for approximately one-fourth of the total products formed during the reaction of a cotton seedling extract with 13-hydroperoxylinolenic acid.

The mol wt of hydroperoxide isomerase has not been reported in the literature. Heimann and Klaiber (11) found that hydroperoxide isomerase from defatted rye seed was a high mol wt protein that eluted with the void volume of a Sephadex G-150 gel filtration column, indicating a mol wt in excess of 150,000. Our gel filtration experiments showed a mol wt of approximately 250,000 for cotton hydroperoxide isomerase. Since protein shape also influences the elution properties of proteins during gel filtration, this result should be taken only as a working value until more rigorous experiments are undertaken, *i.e.* sedimentation-velocity centrifugation of a homogeneous enzyme preparation. The fact that hydroperoxide isomerase and hydroperoxide cyclase activities eluted with the same protein fraction is consistent with observations we have made of the two enzyme activities from other sources, namely that they have never been separated. With enzyme preparations from flaxseed, the two activities were found in the same proportions in the precipitates formed by ammonium sulfate fractionation and both activities were lost at the same rate with mild heat treatment.

If isomerase and cyclase activities are associated with the same protein, it could be argued that hydroperoxide cyclase provides the functional product (12-oxo-PDA) and that hydroperoxide isomerase serves to convert extraneous 9- and 13-hydroperoxides of linoleic acid to innocuous products. 12-Oxo-PDA is structurally similar to the prostaglandins, which regulate physiological processes in mammalian metabolism, and it may be that 12-oxo-PDA has a similar role in plants. However, the demonstration of an acute physiological response by plants to 12-oxo-PDA, either *in vitro* or *in vivo*, has thus far remained elusive. Whatever the function of these oxygenated fatty acids may be, it is clear that the enzymes which catalyze their synthesis are highly active in young seedlings, and that the products likely have a prominent role, as yet not defined, during the early days of plant development.

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