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Lipoxygenase and Hydroperoxide Lyase in Germinating Watermelon Seedlings[1]

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ABSTRACT

Lipoxygenase (EC 1.13.1.13) was found in seedlings of *Citrullus lanatus* (Thunb.) Matsum. and Nakai (watermelon). The enzyme has pH optima of 4.4 and 5.5 and is inhibited by 0.2 mM nordihydroguaiaretic acid. It is present in two functional units with estimated molecular weights of 120,000 and 240,000, respectively.

A new enzyme, tentatively termed hydroperoxide lyase, has been partially purified from watermelon seedlings. The enzyme, located principally in the region of the hypocotyl-root junction, catalyzes the conversion of 13-L-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid to 12-oxo-*trans*-10-dodecenoic acid and hexanal. The hydroperoxide lyase enzyme from watermelon has a molecular weight in excess of 250,000, a pH optimum in the range of 6 to 6.5, and is inhibited by *p*-chloromercuribenzoic acid. Its presence has also been demonstrated in other cucurbits.

The maximum activity of both enzymes occurs on the 6th day of germination. The identification of the products of the hydroperoxide lyase reaction suggests that lipoxygenase and hydroperoxide lyase may be involved in the conversion of certain polyunsaturated fatty acids to traumatic acid (*trans*-2-dodecenedioic acid).

Lipoxygenase catalyzes the incorporation of molecular O_2 into certain polyunsaturated fatty acids having a *cis,cis* 1,4-pentadiene system to form a fatty acid hydroperoxide. The enzyme is present in a variety of higher plants (2) as well as in lower forms of plant life such as *Chlorella pyrenoidosa* (28). Although the existence of lipoxygenase in plants has been known for more than 40 years, the function of the hydroperoxide product has not yet been established.

In 1966 Zimmerman (26, 27) reported the presence of hydroperoxide isomerase in flaxseed which converted linoleic acid hydroperoxide to an α-ketol fatty acid. Later this enzyme was found to be present in corn germ (7) and barley flour (9). These two sources also possessed hydroperoxide isomerases whose products were γ-ketols. Esselman and Clagett (5), working with alfalfa seeds and seedlings, adopted the name lipohydroperoxidase for an enzyme which differed from the hydroperoxide isomerases of corn germ and barley flour, but which also catalyzed the formation of a γ-ketol from linoleic acid hydroperoxide. Heimann *et al.* (14) showed that extracts from oats possessed a hydroperoxide isomerase which converted the hydroperoxide to a hydroxy-epoxy fatty acid. Galliard et al. (6), using extracts of potato tuber, reported still another enzyme which could utilize linoleic acid hydroperoxide. Its product was a butadienyl-vinyl ether fatty acid.

This paper is the first report of the presence of lipoxygenase in germinating watermelon seedlings. Also present is an enzyme which utilizes the hydroperoxide product of the watermelon lipoxygenase reaction. This enzyme from watermelon seedlings was originally reported to be a hydroperoxide isomerase (29), based on its ability to disrupt the conjugated double bond system of the dienoic hydroperoxide and the resulting loss of absorbance at 234 nm. It is now clear that this enzyme converts the hydroperoxide to a 12-carbon, monoenoic ω-oxoacid and hexanal. This new enzyme of fatty acid hydroperoxide metabolism has been tentatively termed hydroperoxide lyase.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Watermelon seeds (*Citrullus lanatus* (Thunb.) Matsum. and Nakai, formerly *C. vulgaris* Schrad. var. Charleston Gray) were soaked overnight in water and placed between two sheets of moist paper toweling 2 cm from the top. To prevent moisture loss, the moist toweling with seeds was placed on a sheet of waxed paper, rolled up, and placed upright in a beaker of water. This was considered to be time zero when determining developmental times. The seeds were germinated in the dark at 30 C and 80% relative humidity.

Preparation of Crude Extracts. Six-day-old watermelon seedlings were separated into cotyledons and the hypocotyl-root section. The tissues were cut into small pieces with a razor blade, transferred to a mortar, frozen in liquid N_2, and ground to a powder with a pestle. Enzymes were extracted by grinding the tissue with 0.05 M potassium phosphate buffer, pH 6, at 4 C. For cotyledons, the ratio of buffer to fresh weight was 4:1 (v/w) and for the hypocotyl-root section, the ratio was 2:1 (v/w). The homogenate was passed through two layers of cheesecloth and centrifuged for 10 min at 12,000*g*. The supernatant was clarified by passing it through two layers of cheesecloth to exclude lipid material which separated during centrifugation.

Determination of Watermelon Lipoxygenase Specificity. An 8 mM linoleic acid (Nu Chek Prep)[3] substrate solution, prepared according to the method of Surrey (24), was converted to a hydroperoxide using a crude extract of watermelon cotyledons that was substantially free of hydroperoxide-metabolizing enzymes. The incubation mixture consisted of 500 ml of 0.05 M potassium phosphate buffer (pH 6), 20 ml of 8 mM linoleic acid substrate solution, and 1 ml of the crude cotyledon extract. The reaction mixture was incubated for 1 hr at room temperature,

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acidified to pH 2, 200 ml of saturated ammonium sulfate added, and extracted overnight with 400 ml of petroleum ether. After concentration of the ether extract, the products were esterified with diazomethane and purified by TLC (Adsorbosil-5-P, Applied Science Laboratories, Inc.) using petroleum ether-ethyl ether-glacial acetic acid (50:50:1, v/v) as the developing solvent. The hydroperoxide product was visualized under UV light, eluted from the gel with ethyl ether, reduced with sodium borohydride, and hydrogenated. The methyl hydroxystearate product was purified by TLC with the solvent system described above, and a mass spectrum was obtained.

Isolation of Products Formed from Linoleic Acid in Extracts of Watermelon Seedlings. The products of linoleic acid and extracts of watermelon seedlings were prepared by adding 15 ml of a cotyledon extract and 60 ml of an extract of the hypocotyl-root tissue to 25 ml of 8 mM linoleic acid substrate solution in 500 ml of 0.05 M potassium phosphate buffer, pH 6. After incubating for 2 hr at room temperature, the mixture was acidified to pH 2 and 200 ml of saturated ammonium sulfate solution were added. Two 24-hr extractions were made with 300 ml of petroleum ether. The petroleum ether extract was dried over anhydrous sodium sulfate, concentrated under reduced pressure, and the products were esterified. The mixture was applied to TLC plates and developed three times in a solvent consisting of petroleum ether-ethyl ether-glacial acetic acid (85:15:1), v/v). Products were visualized under UV light or after spraying a portion of the plate with 70% sulfuric acid saturated with potassium dichromate and charring at 180 C. Products were eluted from the gel with ethyl ether.

Characterization of Products. Fatty acid methyl esters were obtained by treatment with diazomethane. Hydroperoxide and carbonyl groups were reduced to hydroxyl groups by adding 3 to 5 mg of sodium borohydride to the sample dissolved in 2 ml of 95% ethanol. After 15 min at room temperature, two drops of concentrated HCl were added, the ethanol solvent was evaporated, and the reduced sample was redissolved in ethyl ether. Double bonds were reduced by hydrogenating the sample in 95% ethanol for 30 min at room temperature with 5 mg of 10% palladium on Norite as catalyst. TMS[4] ether derivatives of hydroxyl compounds were prepared by reacting the sample with 0.2 ml of Tri-Sil (hexamethyldisilazane-trimethylchlorosilane-pyridine, 2:1:10 (v/v), Pierce Chemical Co.) for 7 min at room temperature. After evaporation of the pyridine solvent under a stream of N_2, the sample was redissolved in ethyl ether.

Infrared spectra were determined on a Perkin Elmer Model 337 spectrophotometer using microliquid cells with a 0.50 mm path length and CCl_4 as solvent. Analysis of fatty acid products by gas chromatography was accomplished on a column (1.5 m × 3 mm o.d.) containing 3% SE-30 on 100/200 mesh Varaport 30 (Varian Aerograph) with temperature programming from 70 to 130 C at 2 C per min. Also used was a column (3 m × 3 mm o.d.) with 10% Silar 9CP (Applied Science Laboratories, Inc.) on 80/100 mesh Gas Chrom Q, operated isothermally at 190 C. Mass spectra were recorded with a Varian/MAT CH-5DF mass spectrometer at an electron potential of 70 ev and a probe temperature ranging from 25 to 150 C.

The position of double bonds was determined by oxidative ozonolysis. Ozone was passed through a solution of the sample in petroleum ether at −60 C for 2 min. The solution was flushed with N_2, the petroleum ether evaporated, and the ozonide cleaved by heating at 80 C for 1 hr with 0.5 ml of performic acid. Performic acid was prepared from 1 ml of 37% (v/v) hydrogen peroxide and 2 ml of 87% (w/v) formic acid. The products were extracted twice with petroleum ether, esterfied, and the retention times determined on both the SE-30 and Silar 9CP columns.

Hexanal was measured by GLC on both a glass column (1.8 m × 2 mm i.d.) containing 15% DEGS on 100/200 mesh Chromosorb W-HP and a column (0.6 m × 4.7 mm i.d.) containing 3% SE-30 on 100/120 mesh Gas Chrom Q. Both were operated isothermally at 70 C. The formation of hexanal was measured by placing 130 ml of a solution containing 0.05 M potassium phosphate buffer (pH 6.2), 27 μM linoleic acid, and 525 μg of pentanal as an internal standard into the outer area of a 250-ml flask with a center well. The center well was filled with 20 ml of a watermelon hypocotyl-root extract (2 mg/ml protein). The flask was sealed with a septum and 10 min were allowed for equilibration of gases. The reaction was initiated by introducing 0.2 ml of a watermelon cotyledon extract (1 mg/ml protein) through the septum and into the reaction mixture without disturbing the hypocotyl-root extract in the center well. Headspace aliquots of 2 ml were taken at various time intervals and injected onto the DEGS column. After 15 min the flask was shaken to introduce the hypocotyl-root extract from the center well, followed by the sampling of 2-ml headspace aliquots. Hexanal concentration was calculated from the concentration of the internal standard, pentanal. Equivalent weights of pentanal and hexanal in solution gave equal responses on GLC when headspace aliquots were analyzed.

Enzyme Purification. Sixty ml of a crude watermelon hypocotyl-root extract were fractionated by ammonium sulfate precipitation at 60% saturation and centrifuged for 10 min at 12,000*g*. The pellet was resuspended in 2 ml of buffer and applied to a Sephadex G-200 column (2.1 × 95 cm). The protein was eluted with 0.1 M potassium phosphate buffer, pH 6.2, containing 0.1 mM dithioerythritol and 0.3 mM EDTA, and collected in 2-ml fractions. Fractions were assayed for lipoxygenase and hydroperoxide lyase. Molecular weights were estimated by comparison with the elution volumes of proteins with known mol wt.

Enzyme Assays. Lipoxygenase activity was determined by measuring the conjugated diene absorption of the hydroperoxide at 234 nm. The reaction mixture contained 2.9 ml of 0.05 M potassium phosphate buffer (pH 6), 0.02 ml of 8 mM linoleic acid substrate solution, and 0.01 to 0.10 ml of enzyme solution. The reaction, conducted at room temperature, was initiated by the addition of the substrate solution.

Hydroperoxide lyase was assayed by the loss in absorption at 234 nm by the hydroperoxide. Hydroperoxide substrate solution for this assay was prepared by incubating 0.6 ml of 8 mM linoleic acid substrate solution with 1.2 mg of soybean lipoxygenase (8200 units/mg, Sigma Chemical Co.) in 30 ml of distilled H_2O for 30 min. The final reaction mixture contained 0.5 ml of the hydroperoxide substrate solution, 0.02 to 0.10 ml of enzyme solution, and 0.1 M potassium phosphate buffer, pH 6, to provide a final volume of 3 ml. In some experiments 10 mM KCN was added to the reaction mixture. This greatly reduced the absorbance of the aldehyde product by converting it to a nonabsorbing cyanohydrin compound. The reaction was run at room temperature and was initiated by the addition of the enzyme solution.

Protein was determined by the Lowry method (18).

RESULTS

Specificity of Watermelon Lipoxygenase. The mass spectrum of the reduced, saturated hydroperoxide product of watermelon lipoxygenase and linoleic acid is shown in Figure 1. The ion fragments at m/e 211, 214, and 243 are characteristic of methyl-13-hydroxystearate (22). The absence of the series of ion fragments at m/e 155, 158, and 187, which is characteristic of the 9-hydroxy isomer, indicates that watermelon lipoxygenase is specific for O_2 attack at carbon 13 of linoleic acid.

Characterization of Products Formed from Linoleic Acid in Extracts of Watemelon Seedlings. The methyl esters of the fatty

[4] Abbreviation: TMS; trimethylsilyl.

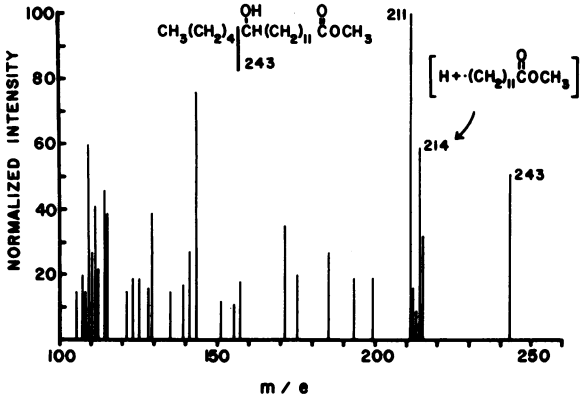

FIG. 1. Partial mass spectrum of methyl-13-hydroxystearate resulting from reduction of the hydroperoxide product of linoleic acid and watermelon lipoxygenase.

acid products resulting from the incubation of linoleic acid substrate with a crude extract of 6-day-old, etiolated watermelon cotyledons and hypocotyls were separated by TLC (Fig. 2). One of the products, labeled H, was linoleic acid hydroperoxide, based on its R_F and UV absorption at 234 nm. The other major products, A, B, C, and D, in order of increasing R_F, were eluted from the silica gel and characterized.

An IR spectrum of the methyl ester of product A (Fig. 3) showed absorbances at 3590 cm^{-1} (hydroxyl), 3010 cm^{-1} (unsaturation), and 900 cm^{-1} (epoxide) in addition to the ester carbonyl at 1740 cm^{-1}. There was no absorbance at 970 cm^{-1}, denoting the absence of *trans* double bonds. The compound had no characteristic absorbance in the 234 nm region, indicating no double bond conjugation. A mass spectrum of the TMS ether derivative showed a large peak at m/e 285, which placed the hydroxyl group at carbon 11 (Fig. 4). Absence of an m/e 215 peak (cleavage on the other side of the TMS group) indicated that the double bond was α,β to the hydroxyl (16). A parent peak at m/e 398, and confirmed by M-15 and M-31 peaks, was consistent with an 18-carbon monoenoic, epoxy-hydroxy fatty acid. Treatment with periodate caused cleavage, as evidenced by GLC analysis, indicating that the epoxide is adjacent to the hydroxyl group. The compound was thus identified as 11-hydroxy-12,13-epoxy-*cis*-9-octadecenoic acid.

Product B showed IR absorption at 3620 cm^{-1} (hydroxyl), 3015 cm^{-1} (unsaturation), and at 983 and 950 cm^{-1} (*cis-trans* conjugation). The compound absorbed strongly in the UV region at 234 nm, characteristic of two double bonds in conjugation. A mass spectrum of the TMS ether derivative, purified with an OV17 GLC column on line with the mass spectrometer, gave a parent peak at m/e 382 (Fig. 5), consistent with an 18-carbon dienoic, hydroxy fatty acid. A large peak at m/e 311 and a lesser peak at m/e 225 placed the hydroxyl at carbon 13 (16). The compound was identified as 13-hydroxy-*cis*-9-*trans*-11-octadecadienoic acid.

Product C was identified as an aldehyde on the basis of its reaction with aldehyde-sensitive reagents, basic fuchsin and 4-amino-5-hydrazino-1,2,4-triazole-3-thiol (21), on thin layer plates. An IR spectrum of product C (Fig. 6) showed the presence of unsaturation (3020 cm^{-1}) as well as the characteristic aldehyde absorption at 2810 and 2740 cm^{-1}. The ester carbonyl was retained as demonstrated by the 1740 cm^{-1} absorption. The 970 cm^{-1} absorption showed the presence of a *trans* double bond. Absorption at 1690 cm^{-1} indicated α,β unsaturation of the aldehyde (19). An absorption maximum at 222 nm by compound C also suggested an α,β unsaturated carbonyl.

When the methyl ester of product C was analzyed by GLC on an SE-30 column, it was apparent from its retention time that it had a much shorter chain length than did the other products. To determine the chain length, product C was reduced with sodium borohydride and the TMS ether derivative prepared. The derivative was purified on an SE-30 GLC column equipped with a 10:1 splitter, and a mass spectrum obtained from the isolated compound (Fig. 7). The spectrum was typical of that for an ω-

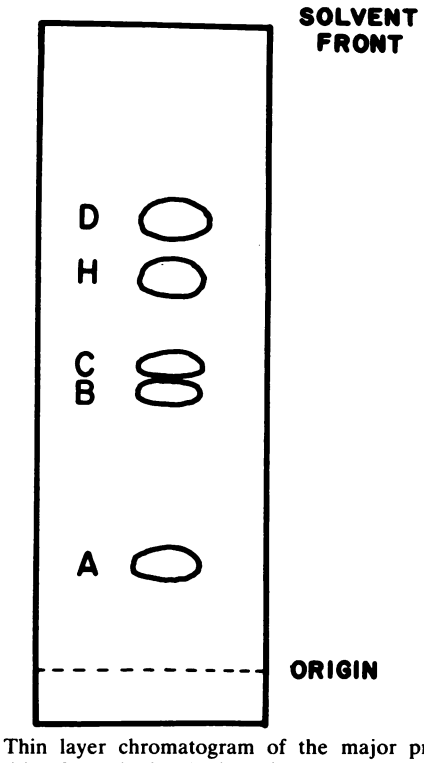

FIG. 2. Thin layer chromatogram of the major products (methyl esters) resulting from the incubation of linoleic acid with an extract of watermelon cotyledons and hypocotyls. Product A, 11-hydroxy-12,13-epoxy-*cis*-9-octadecenoic acid; product B, 13-hydroxy-*cis*-9-*trans*-11-octadecadienoic acid; product C, 12-oxo-*trans*-10-dodecenoic acid; product D, 13-oxo-*cis*-9-*trans*-11-octadecadienoic acid; and product H, 13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid. Products A, B, and D were formed nonenzymically. Petroleum ether-ethyl ether-glacial acetic acid, 85:15:1 (v/v), three developments.

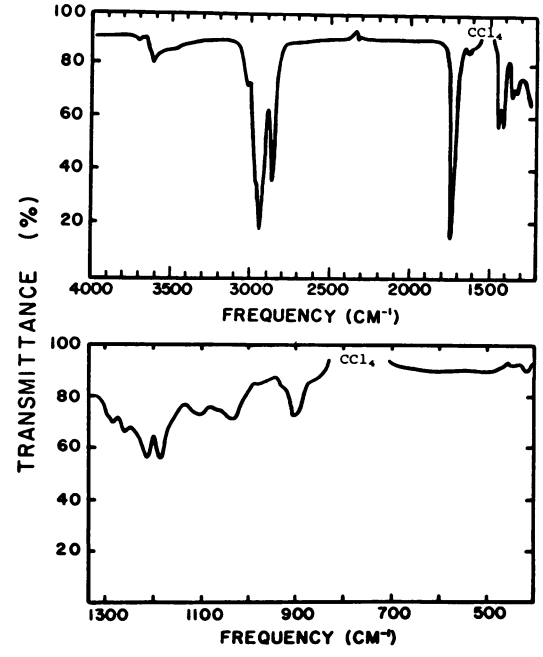

FIG. 3. Infrared spectrum of the methyl ester of product A. This product resulted from the incubation of linoleic acid with an extract of watermelon cotyledons and hypocotyls. The solvent was CCl_4.

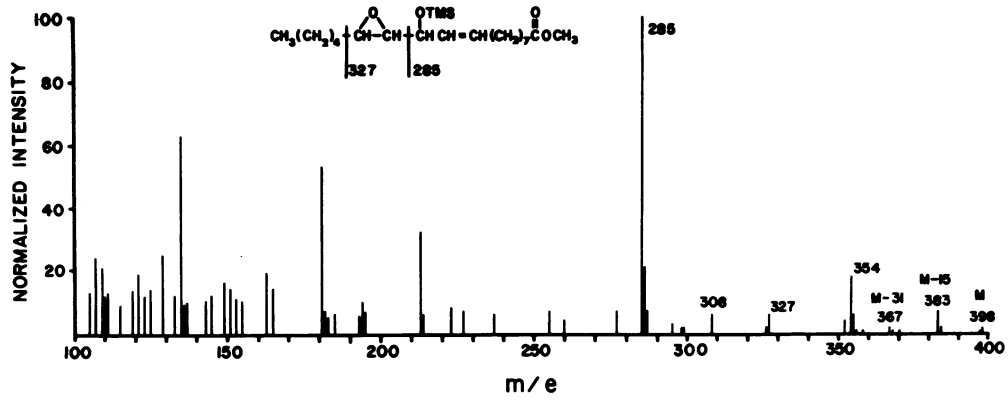

FIG. 4. Partial mass spectrum of the TMS ether derivative of product A (methyl ester). This product resulted from the incubation of linoleic acid with an extract of watermelon cotyledons and hypocotyls.

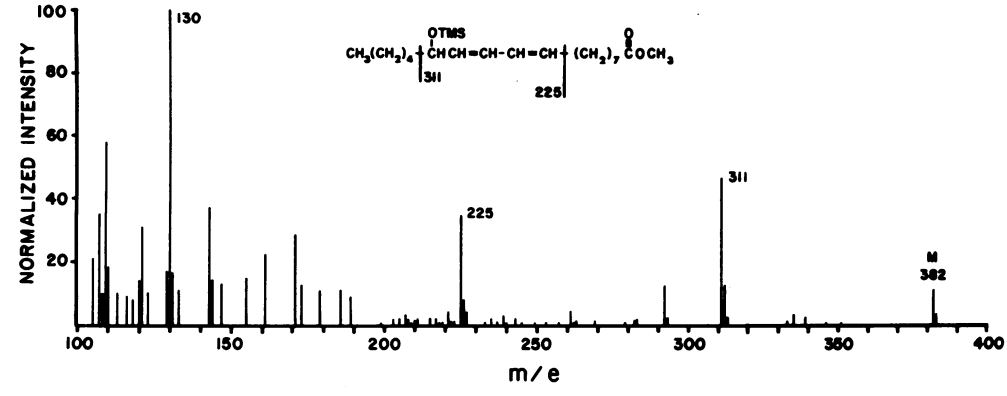

FIG. 5. Partial mass spectrum of the TMS ether derivative of product B (methyl ester). This product resulted from the incubation of linoleic acid with an extract of watermelon cotyledons and hypocotyls.

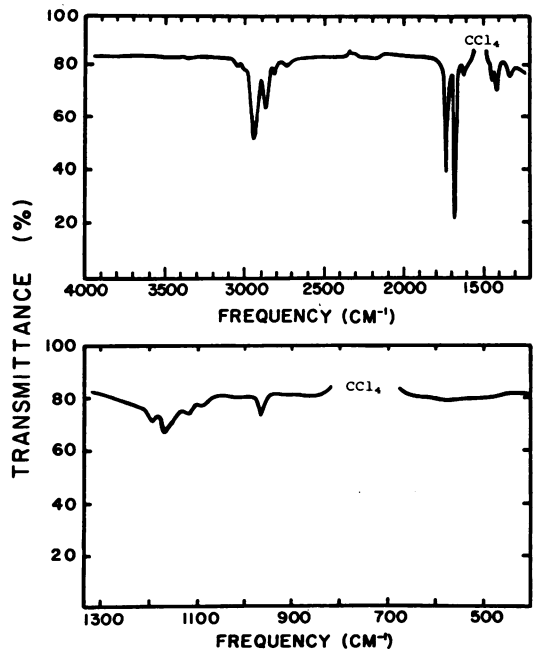

FIG. 6. Infrared spectrum of the methyl ester of product C. This product resulted from the incubation of linoleic acid with an extract of watermelon cotyledons and hypocotyls. The solvent was CCl_4.

hydroxy fatty acid (3). The parent peak at m/e 300 was verified by characteristic peaks at M-15 and M-47. The mol wt of 300 established that the compound had a chain length of 12 carbons and one double bond. In addition to the information provided by the IR and UV spectra, oxidative ozonolysis confirmed the position of the double bond. Analysis of the methyl ester of the ozonolysis product by gas chromatography on both an SE-30 and a Silar 9CP column showed it to be a 10-carbon dicarboxylic acid. Therefore, the position of the double bond was between carbons 10 and 11, and product C was concluded to be 12-oxo-*trans*-10-dodecenoic acid.

A mass spectrum of the nonderivatized methyl ester of compound C, purified on an SE-30 column, is shown in Figure 8. Unexplained peaks were present in this mass spectrum. A peak at m/e 224 was present rather than a parent peak at m/e 226 as expected. Although the evidence described earlier confirmed conclusively the structure of product C, the difference of two

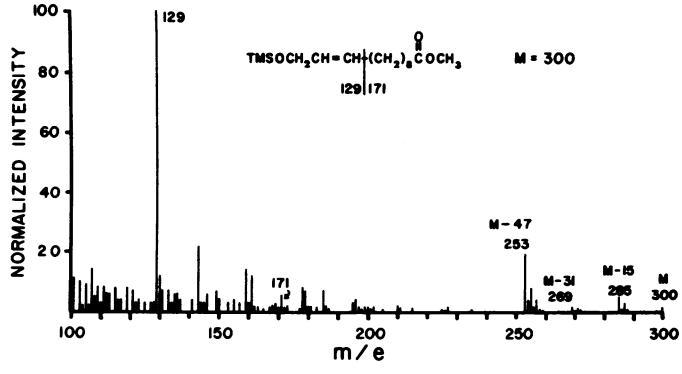

FIG. 7. Partial mass spectrum of the TMS ether derivative of product C (methyl ester) after reduction with $NaBH_4$. Product C resulted from the incubation of linoleic acid with an extract of watermelon cotyledons and hypocotyls.

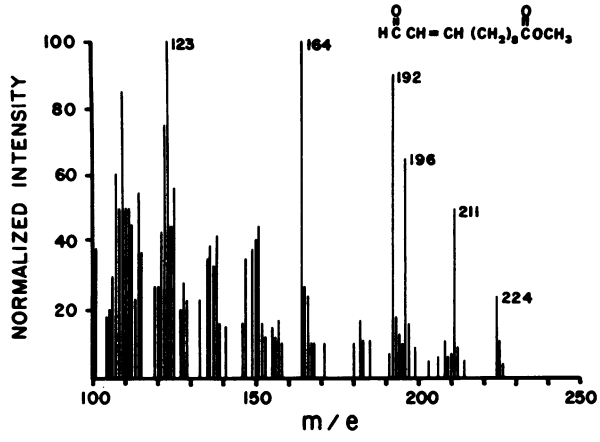

FIG. 8. Partial mass spectrum of the nonderivatized methyl ester of product C. This product resulted from the incubation of linoleic acid with an extract of watermelon cotyledons and hypocotyls.

mass units observed in the mass spectrum was disturbing. It appears that long chain α,β unsaturated aldehydes undergo unusual rearrangements during the ionization process. The peak at m/e 224 could result from loss of 2 hydrogen atoms during rearrangement to form a cyclic furan ion. The peak at m/e 192 could have resulted from loss of CH_3OH from this ion. Other unusual peaks were at m/e 211 (M-15), m/e 196, and m/e 164. Loss of formaldehyde (M-30) followed by loss of methanol (M-30-32) could explain these latter two peaks.

Product D was characterized as a conjugated ketodiene compound on the basis of its R_F, its UV absorption at 278 nm, and its IR spectrum, which showed absorbances at 1740 cm^{-1} (ester carbonyl), 1690 and 1638 cm^{-1} (characteristic of carbonyl conjugation), and at 990 and 950 cm^{-1} (*cis-trans* conjugation). The properties of ketodienes have been described by Vioque and Holman (25). Since watermelon lipoxygenase attacks linoleic acid solely at position 13, it is likely that product D is 13-oxo-*cis*-9-*trans*-11-octadecadienoic acid.

To determine which products were formed enzymically from the hydroperoxide, pure linoleic acid hydroperoxide was prepared from soybean lipoxygenase and incubated with an extract of watermelon cotyledons and hypocotyls which had been heat-treated for 10 min at 100 C. The heat-treated extract was able to convert the hydroperoxide to a mixture of products A, B, and D, demonstrating that the only enzymic reation of linoleic acid hydroperoxide with watermelon extracts was the formation of product C, 12-oxo-*trans*-10-dodecenoic acid. This enzyme has been tentatively designated as hydroperoxide lyase (Fig. 9). In addition to chain cleavage, the enzyme catalyzes the isomerization of a *cis*-9-double bond to a *trans*-10 double bond.

There was no evidence that an α-ketol was present as a reaction product. A purified α-ketol, prepared from flaxseed hydroperoxide isomerase, was nonreactive in the presence of the crude watermelon extract. This indicated that the α-ketol was probably not an intermediate in the lyase reaction. The possibility remains that hydroperoxide isomerase is one component of a large hydroperoxide lyase complex, and that its α-ketol product is accessible to the next enzyme in the complex only if it entered the sequence as a hydroperoxide.

The nonenzymic conversion of linoleic acid hydroperoxide to epoxy-hydroxy, hydroxydiene, and ketodiene compounds was not unexpected. Hamburg has shown that this conversion can be catalyzed by heme-containing compounds (12). To determine whether one of these nonenzymic products may have been the actual substrate for the lyase reaction, each of the three nonenzymic products was isolated from thin layer plates and incubated in the acid form with the crude watermelon extract. Each product was nonreactive, indicating that the true substrate was the hydroperoxide.

The other product of the hydroperoxide lyase reaction was identified as hexanal by comparison of its retention time with the retention time of pure hexanal on two GLC columns, DEGS and SE-30. Mass spectrometry confirmed that the suspect peak material was hexanal. Figure 10 shows the enzymic formation of hexanal with extracts of watermelon seedlings. There was no hexanal formation when a watermelon cotyledon extract, which contained lipoxygenase but not hydroperoxide lyase, was added to a solution of linoleic acid in potassium phosphate buffer, pH 6.2. The absorbance at 234 nm, however, increased rapidly due to the lipoxygenase-catalyzed formation of linoleic acid hydroperoxide. After 15 min an extract of watermelon hypocotyl-root, containing the lyase, was added to the reaction mixture. An immediate decrease in absorption at 234 nm (loss of the conjugated diene hydroperoxide) was accompanied by a rapid increase in hexanal concentration. When a heat-treated extract of watermelon hypocotyl-root was added, there was no decrease in absorption at 234 nm and no hexanal formation. This experiment verified that the spectrophotometric assay at 234 nm was a valid assay for watermelon hydroperoxide lyase.

Although linoleic acid was utilized for all of the product

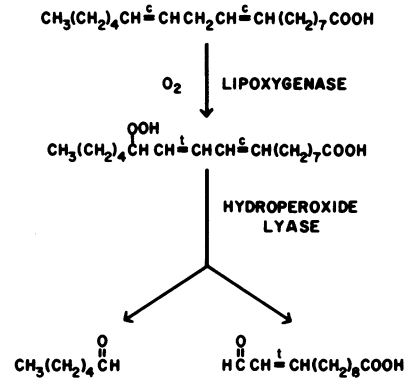

FIG. 9. Reactions catalyzed by watermelon lipoxygenase and hydroperoxide lyase.

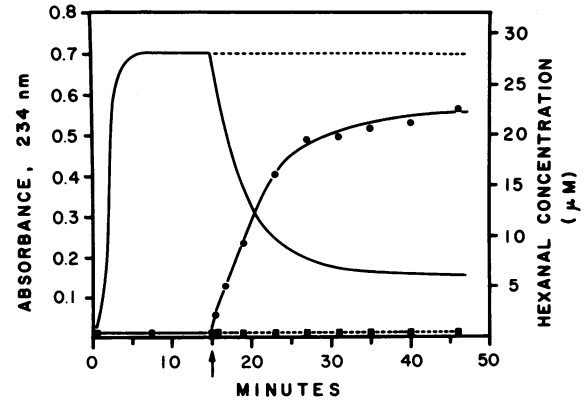

FIG. 10. Reaction of linoleic acid with a watermelon cotyledon extract, followed by addition of a watermelon hypocotyl-root extract at 15 minutes (arrow). Absorbance at 234 nm due to presence of the hydroperoxide (——); absorbance at 234 nm after the addition of a heat-treated watermelon hypocotyl-root extract (- - -); hexanal concentration (●——●); hexanal concentration after the addition of a heat-treated watermelon hypocotyl-root extract (■- - -■). The 150-ml reaction mixture contained 50 mM potassium phosphate buffer, pH 6.2, 27 μM linoleic acid, 0.2 mg of watermelon cotyledon protein, and 40 mg of watermelon hypocotyl-root protein. In addition, the aliquot used for the spectrophotometric assay contained 10 mM KCN.

characterization, linolenic acid hydroperoxide was also metabolized in a similar manner by a watermelon crude extract.

pH Optima of Watermelon Lipoxygenase and Hydroperoxide Lyase. A crude extract of cotyledons from 5-day-old etiolated watermelon seedlings was used to determine the pH optimum of lipoxygenase (Fig. 11). The lipoxygenase exhibited two pH optima, one at pH 4.4 and the other at pH 5.5. Hydroperoxide lyase extracted from the hypocotyl-root had its maximum activity in the range of pH 6 to 6.5 (Fig. 11).

Enzyme Purification and Molecular Weight Estimation. The protein from an extract of watermelon hypocotyl-root which precipitated at 60% saturation with ammonium sulfate was applied to a Sephadex G-200 column. The elution profile of hydroperoxide lyase and lipoxygenase is shown in Figure 12.

Hydroperoxide lyase was eluted near the void volume, and consequently a precise estimation of its mol wt was not possible, except to say that it was in excess of 250,000. The partially purified enzyme was not inhibited by 10 mM iodoacetamide or 10 mM cyanide. In the presence of 0.1 mM *p*-chloromercuribenzoate the enzyme was inhibited 83%, suggesting that certain sulfhydryl groups must be intact to preserve enzyme activity. The enzyme was inactive in 7 M urea. Table I shows the purification of hydroperoxide lyase from watermelon hypocotyl-root tissue. A 42-fold purification of the enzyme from crude extract was obtained.

Since lipoxygenase has not previously been reported in watermelon, further investigation of its properties was made. Watermelon lipoxygenase exhibited two peaks of activity on the Sephadex G-200 column (Fig. 12). For purposes of identification, the second peak (lower mol wt) is referred to as lipoxygenase-1 and the first peak (higher mol wt) as lipoxygenase-2. From a plot of V_e/V_0 *versus* the logarithm of the mol wt for known proteins, the mol wt of lipoxygenase-1 was estimated to be 120,000 and the mol wt of lipoxygenase-2 was estimated at 240,000 (Fig. 13). Thus, lipoxygenase-2 appears to consist of 2 subunits of lipoxygenase-1. A dimer of lipoxygenase with such high mol wt has not been reported previously. The enzyme was inhibited 98% in the presence of 0.2 mM nordihydroguaiaretic acid.

Enzyme Development during Germination. Watermelon seeds, germinated in moist paper toweling in the dark, were harvested and separated into two parts, the cotyledons and the remaining hypocotyl-root section, over a period of 10 days. Each part was extracted and assayed for lipoxygenase and hydroperoxide lyase activities. Neither enzyme was present in ungerminated watermelon seed, but after the 3rd day of germination a rapid rise in the activity of both enzymes occurred (Figs. 14 and 15). By the 6th day, both enzymes had reached their maximum activities and declined rapidly thereafter. This dramatic increase and decrease in the activity of the two watermelon enzymes during germination suggests a function in the early stage of plant growth.

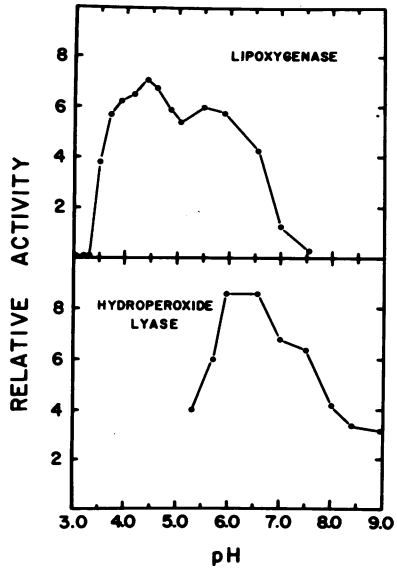

FIG. 11. Activity of watermelon lipoxygenase (top) and hydroperoxide lyase (bottom) as a function of pH. The enzymes were measured spectrophotometrically at 234 nm. The buffers used were 50 mM citrate (pH 3–5.3), 50 mM potassium phosphate (pH 5.5–8), and 10 mM borate (pH 8.4–9).

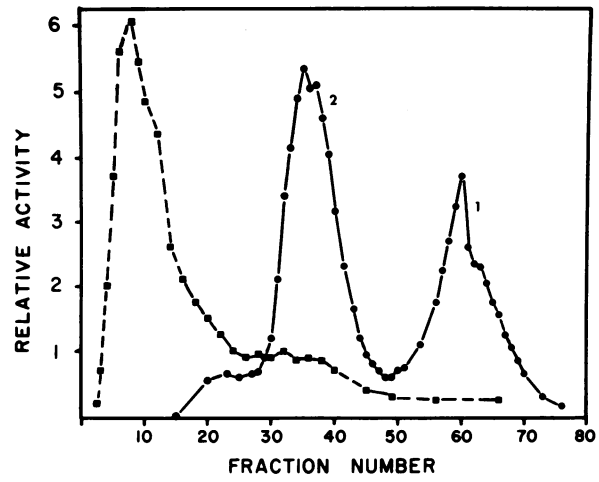

FIG. 12. Separation of hydroperoxide lyase (■- -■) and lipoxygenase (●——●) 1 and 2 from watermelon hypocotyl-root tissue on Sephadex G-200 (2.1 × 95 cm). The elution buffer was 0.1 M potassium phosphate, pH 6.2, containing 0.1 mM dithioerythritol and 0.3 mM EDTA.

Enzyme Distribution within Seedling. The enzyme distribution within 6-day-old etiolated watermelon seedlings was investigated by separating the seedlings into two sections, the cotyledons and the hypocotyl-root section, and extracting with buffer. Almost all of the lipoxygenase, 98%, was located in the cotyledons (Table II). However, the hydroperoxide lyase activity was located principally in the hypocotyl-root section (81%).

Because of the high activity of hydroperoxide lyase in the hypocotyl-root, it was of interest to know its distribution within this section. Table III shows the activities of three different subsections: (*a*) the root, (*b*) a 1-cm section at the junction of root and hypocotyl, and (*c*) the hypocotyl. The highest activity occurred in the 1-cm center section between the hypocotyl and root. In terms of specific activity, the center section was at least 5 times more active than the root and over 10 times more active than the hypocotyl. The reason for the high activity of hydroperoxide lyase in this section is not clear.

Survey of Plants for Hydroperoxide Lyase Activity. Ten plant species in addition to watermelon were surveyed for lyase activity. Of these, only cucumber and cantaloupe, which are taxonomically closely related to watermelon, showed formation of 12-oxo-*trans*-10-dodecenoic acid. Hydroperoxide lyase activity was not detected in barley, corn, flax, green beans, peas, pumpkin, squash, or sunflower.

DISCUSSION

There have been several reports that linoleic and linolenic acid are precursors of short chain volatile aldehydes (1, 8, 13, 15, 23). However, nonvolatile, longer chain products were not investigated in any of these studies. Grosch and Schwarz (10) have shown that linoleic acid is a precursor of volatile and nonvolatile aldehydes in cucumbers. The products identified were hexanal

Table I. *Purification of Hydroperoxide Lyase from Watermelon*

Six-day-old, etiolated hypocotyl-root sections of watermelon seedlings were extracted with 0.05 M potassium phosphate buffer, pH 6. The protein was precipitated at 60% ammonium sulfate saturation, applied to a Sephadex G-200 column (2.1 × 95 cm), and eluted with 0.1 M potassium phosphate buffer, pH 6.2, containing 0.1 mM dithioerythritol and 0.3 mM EDTA.

Fraction	Total protein	Total units[1]	Specific activity	Purification	Recovery
	mg		units/mg		%
Crude extract	125	1290	10.3	1	100
$(NH_4)_2SO_4$ ppt	20	446	22.3	2.2	35
Sephadex G-200 eluate	0.73	308	423	42	24

[1] One unit of activity is defined as 1 μmole of hydroperoxide decomposed per minute at 25 C.

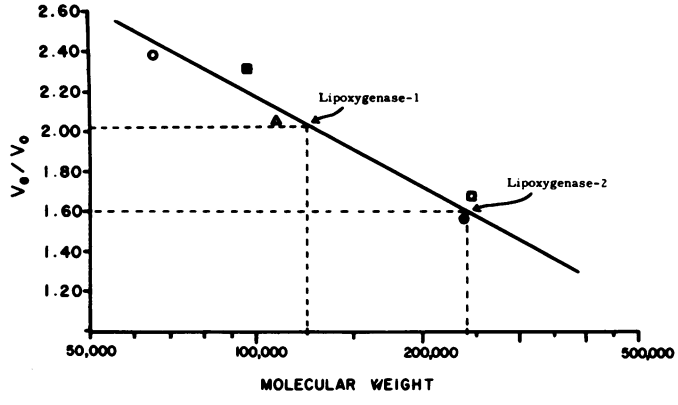

FIG. 13. Estimation of the molecular weights of watermelon lipoxygenase. Hemoglobin (○), hexokinase (■), soybean lipoxygenase (△), pyruvate kinase (●), and catalase (□).

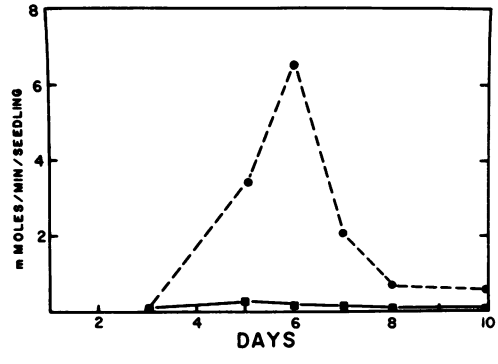

FIG. 14. Watermelon lipoxygenase activity during germination. Cotyledons (●- -●) and hypocotyl (■——■).

and *trans*-2-nonenal. Formation of the latter product was similar to the hydroperoxide lyase reaction in that chain cleavage occurred, followed by isomerization of a *cis*-3 double bond to a *trans*-2 double bond.

The function of 12-oxo-*trans*-10-dodecenoic acid has not been established. Its structure, however, is very similar to that of a compound which is thought to induce cell division. Haberlandt (11) showed the presence of a substance in extracts of injured plant cells that worked in conjunction with a second factor contained in the phloem to induce cell division in uninjured cells of plant tissue. The substance was identified by English *et al.* (4) as the unsaturated dicarboxylic acid *trans*-2-dodecenedioic acid, commonly called traumatic acid due to its suspected role following plant injury.

The conversion of 12-oxo-*trans*-10-dodecenoic acid to traumatic acid would only require an ω-oxoacid dehydrogenase enzyme. Kolattukudy *et al.* (17) have demonstrated the presence of such an enzyme in the epidermal cells of *Vicia faba* L. leaves. The enzyme was able to convert a 16-carbon ω-oxoacid to a dicarboxylic acid with NADP as the preferred cofactor. If a similar enzyme is present in watermelon seedlings, lipoxygenase and hydroperoxide lyase could function in the conversion of linoleic acid to a suspected plant hormone.

Other lines of evidence also make a hormonal role for lipoxygenase an attractive hypothesis. Oelze-Karow and Mohr (20) have demonstrated that lipoxygenase activity in the cotyledons of mustard seedlings is mediated by Pfr present in the hypocotyledonary hook. Under dark conditions lipoxygenase activity in cotyledons increases as the seed germinates. Exposure of the hook to far red light suppresses this increase.

If a similar phytochrome-mediated control of lipoxygenase is operative in the hypocotyl of watermelon seedlings, its signals could be transmitted to the cotyledons, which are known to possess very high lipoxygenase activity, or to the hypocotyl-root section, which also possesses considerable activity. The product of lipoxygenase catalysis, the hydroperoxide, is metabolized by a lyase enzyme localized mainly at the junction between the root and hypocotyl. The high specific activity of the lyase in this region during the early stage of germination could indicate that

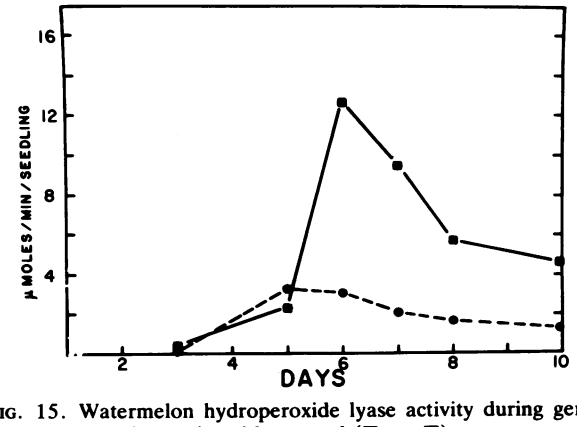

FIG. 15. Watermelon hydroperoxide lyase activity during germination. Cotyledons (●- -●) and hypocotyl (■——■).

Table II. *Lipoxygenase and Hydroperoxide Lyase Distribution within Watermelon Seedlings*

Six-day-old, etiolated watermelon seedlings were separated into the cotyledons and the hypocotyl-root section, extracted with 0.05 M potassium phosphate buffer, pH 6, and assayed for lipoxygenase and hydroperoxide lyase activity.

	Lipoxygenase		Hydroperoxide Lyase	
	Specific activity	Total activity	Specific activity	Total activity
	units[1]/mg	units[1]/section	units[1]/mg	units[1]/section
Hypocotyl-root	107	135 (2)[2]	10	13 (81)[2]
Cotyledons	1550	6500 (98)	0.73	3 (19)

[1] One unit of activity is defined as 1 μmole of hydroperoxide decomposed per minute at 25 C.

[2] Number in parentheses represents percent of total activity.

Table III. *Hydroperoxide Lyase Distribution within a Watermelon Hypocotyl-root Section*

Six-day-old, etiolated watermelon seedlings with cotyledons removed were separated into three sections: the root, a 1-cm section at the junction of root and hypocotyl, and the hypocotyl. Each section was extracted with 0.05 M potassium phosphate buffer, pH 6, and assayed for hydroperoxide lyase activity.

	Hydroperoxide Lyase	
	Specific activity	Total activity
	Units[1]/mg	Units[1]/section
Root	1.4	0.94 (35)[2]
Hypocotyl-root junction	7.0	1.2 (45)
Hypocotyl	0.64	0.55 (20)

[1] One unit of activity is defined as 1 μmole of hydroperoxide decomposed per minute at 25 C.

[2] Number in parentheses represents percent of total activity.

its product is involved in cell differentiation, or that it functions in cell division or cell enlargement in the hypocotyl as it pushes its way through the soil to reach sunlight. Further work is now underway to determine whether the products of watermelon lipoxygenase and hydroperoxide lyase function in some aspect of hormonal activity.

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