α-Oxidation of Fatty Acids in Higher Plants

IDENTIFICATION OF A PATHOGEN-INDUCIBLE OXYGENASE (PIOX) AS AN α-DIOXYGENASE AND BIOSYNTHESIS OF 2-HYDROPEROXYLINOLENIC ACID

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Mats Hamberg, Ana Sanz, and Carmen Castresana

From the Division of Physiological Chemistry II, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden and the Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma, Cantoblanco, E-28049 Madrid, Spain

A pathogen-inducible oxygenase in tobacco leaves and a homologous enzyme from Arabidopsis were recently characterized (Sanz, A., Moreno, J. I., and Castresana, C. (1998) Plant Cell 10, 1523–1537). Linolenic acid incubated at 23 °C with preparations containing the recombinant enzymes underwent α-oxidation with the formation of a chain-shortened aldehyde, i.e., 8(Z),11(Z),14(Z)-heptadecatrienial (83%), an α-hydroxy acid, 2(R)-hydroxy-9(Z),12(Z),15(Z)-octadecatrienioic acid (15%), and a chain-shortened fatty acid, 8(Z),11(Z),14(Z)-heptadecatrienoic acid (2%). When incubations were performed at 0 °C, 2(R)-hydroperoxy-9(Z),12(Z),15(Z)-octadecatrienoic acid was obtained as the main product. An intermediary role of 2(R)-hydroperoxy-9(Z),12(Z),15(Z)-octadecatrienoic acid in α-oxidation was demonstrated by re-incubation experiments, in which the hydroperoxide was converted into the same α-oxidation products as those formed from linolenic acid. 2(R)-Hydroperoxy-9(Z),12(Z),15(Z)-octadecatrienioic acid was chemically unstable and had a half-life time in buffer of about 30 min at 23 °C. Extracts of cells expressing the recombinant oxygenases accelerated breakdown of the hydroperoxide (half-life time, about 3 min at 23 °C), however, this was not attributable to the recombinant enzymes since the same rate of hydroperoxide degradation was observed in the presence of control cells not expressing the enzymes. No significant discrimination between enantiomers was observed in the degradation of 2(R,S)-hydroperoxy-9(Z)-octadecenoic acid in the presence of recombinant oxygenases. A previously studied system for α-oxidation in cucumber was re-examined using the newly developed techniques and was found to catalyze the same conversions as those observed with the recombinant enzymes, i.e. enzymatic α-dioxygenation of fatty acids into 2(R)-hydroperoxides and a first order, non-stereoselective degradation of hydroperoxides into α-oxidation products. It was concluded that the recombinant enzymes from tobacco and Arabidopsis were both α-dioxygenases, and that members of this new class of enzymes catalyze the first step of α-oxidation in plant tissue.

Fatty acid hydroperoxides serve as important intermediates in the oxylipin pathway of fatty acid oxygenation in plants and fungi (1–4). Further metabolism of the hydroperoxide derivatives of linoleic and linolenic acids results in the formation of fatty acid epoxides and epoxy alcohols (5–7), dihydroxy alcohols (8, 9), short-chain aldehydes (10, 11), and divinyl ethers (12–15). One specific hydroperoxide isomer, i.e. the 13(S)-hydroperoxide derivative of linolenic acid, is converted into jasmonic acid by a series of reactions catalyzed by allene oxide synthase, allene oxide cyclase, reductase, and β-oxidation enzymes (2). This pathway is of biological importance in plants because it produces compounds which are involved in defense reactions against insects and other phytopathogens (16), in mechanical responses such as tendril coiling (17), and pollen development (18).

A variety of conditions, such as mechanical perturbation, osmotic stress, attack by plant pathogens and wounding, elicit increased formation of jasmonates and other biologically active oxylipins in plant leaves (19). This is partly a consequence of liberation of free linolenic acid from its esterified forms (20, 21) but may also depend on increased levels of enzymes catalyzing hydroperoxide formation and metabolism. In a recent study, tobacco leaves were found to accumulate a 75-kDa protein in response to bacterial infection (22). This protein, as well as a protein from Arabidopsis showing a 75% homology in amino acid sequence, were expressed in insect cells and found to cause uptake of molecular oxygen in the presence of polysaturated fatty acids such as linolenic acid, linoleic acid, and arachidonic acid. Interestingly, the tobacco enzyme, called “pathogen-inducible oxygenase” (PIOX),1 showed significant homology to prostaglandin-endoperoxide H synthases-1 and -2 present in animal tissue (22).

The present study was carried out with the aim of identifying the catalytic function of the pathogen-induced oxygenase from tobacco leaves and its homologous enzyme from Arabidopsis. Evidence will be presented that both enzymes are fatty acid α-dioxygenases which catalyze conversion of linolenic acid and other fatty acids into their 2(R)-hydroperoxy derivatives. The mode of degradation of these unstable hydroperoxides into chain-shortened aldehydes and other α-oxidation products has also been studied.

1 The abbreviations used are: PIOX, pathogen-inducible oxygenase; BHT, 2,6-di-tert-butyl-4-methylphenol; 9(S)-HPOD, 9(S)-hydroperoxy-10(E),12(Z)-octadecadienoic acid; BSTFA, bis(trimethylsilyl)trifluoroacetamide; GLC, gas-liquid chromatography; GC-MS, gas-liquid chromatography-mass spectrometry; MC, (−)-menthoxy-carbonyl; MO, O-methyloxime; Me, Si, trimethylsilyl; RP-HPLC, reversed phase high performance liquid chromatography.

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EXPERIMENTAL PROCEDURES

Labeled Fatty Acids—[1-14C]Linolenic, [1-13C]Linoleic, and [9,10,12-3H]Linoleic acids were purchased from NEN Life Science Products Inc. (Boston, MA). Dilution with unlabeled materials (Nu-Chek-Prep, Elysian, MN) followed by purification by SiO2 chromatography afforded specimens having specific radioactivities of 8.9, 3.8, and 184 kBq/μmol, respectively. In the same way, [9,10,12,13,16-18]H]Linoleic acid (American Radiolabeled Chemicals, St. Louis, MO) was diluted with unlabeled linolenic acid and purified to make a specimen having a specific radioactivity of 22.2 kBq/μmol.

2Rl- and 2R,S-Hydroxylinolenic Acid—Seeds of Thymus vulgaris (23) were ground in an electric coffee mill and the powder (25 g) was extracted under an argon atmosphere for 2 h with hexane (250 ml) containing BHT (12 ppm) in a Soxhlet apparatus. The oil (8.5 g) was subjected to methanolysis and fractionated by SiO2 open column chromatography. Elution with diethyl ether/hexane (7:3, v/v) afforded methyl 2-hydroxylinolenate (10.0 g) containing BHT (12 ppm) in a Soxhlet apparatus. The oil (8.5 g) was rapidly extracted with diethyl ether. The ether phase was dried over MgSO4 and taken to dryness at 25 °C. Analysis of the specimen by radio-HPLC showed a radiochemical purity in excess of 95%. The specific radioactivity was 32 kBq/μmol (the drop in specific activity compared with that of the starting material was due to a slight chromatographic separation between nitroso- and unlabeled molecules in the RP-HPLC purification step resulting in partial loss of the "H compound."

The residual 2-hydroxylinolenic acid was dissolved in dry acetone and stored at -25 °C. A suspension of this material was added to the reaction mixture in order to maintain oxygen saturation and to effect rapid dispersion of the introduced oleate dianion. Material obtained by extraction with diethyl ether was analyzed by RP-HPLC using solvent system IV. Three main peaks were observed, i.e., 2-hydroperoxyoleic acid (13.0 ml effluent), 2-hydroxyoleic acid (13.8 ml), and oleic acid (27.1 ml). Effluent containing 2-hydroperoxyoleic acid was collected and rapidly extracted with diethyl ether. The ether phase was washed with water and taken to dryness in vacuo. The residual 2-hydroperoxyoleic acid was dissolved in dry acetone and stored at -25 °C. Analysis of the specimen by radio-HPLC showed a radiochemical purity in excess of 95%. The specific radioactivity was 32 kBq/μmol (the drop in specific activity compared with that of the starting material was due to a slight chromatographic separation between nitroso- and unlabeled molecules in the RP-HPLC purification step resulting in partial loss of the "H compound."

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For incubations with [9,10-3H]oleic acid (100 μM) and hydroperoxide (30–264 μM) subsequently, [9,10-3H]oleic acid (100 μM) and hydroperoxide (30–264 μM) were added and stirring continued for 15 min. The reaction products were extracted with diethyl ether, and the material obtained was analyzed by RP-radio-HPLC.

Preparation of 2(R)-Hydroperoxy-9(Z),12(Z),15(Z)-octadecatrienoic Acid—Linolenic acid (5.9 mg; concentration, 152 μM) was stirred at 0 °C for 30 min with a suspension (140 μl) of the 48,000 × g particle fraction of homogenate of cucumber. The mixture was acidified to pH 4 and rapidly extracted with 2 volumes of diethyl ether. The material obtained was analyzed by RP-radio-HPLC.
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tained following evaporation of the solvent was suspended in HPLC mobile phase (0.4 ml). After centrifugation, aliquots of 0.1 ml were subjected to RP-HPLC using solvent system 1 at a flow rate of 2 ml/min. Effluent containing the hydroperoxide (37.0–39.4 ml) was immediately extracted with diethyl ether and the solution dried over MgSO4. Hydroperoxide was eluted as its salt (3.6–6.0 ml effluent), well separated from the main product of hydroperoxide breakdown, i.e. 8(Z),11(Z),14(Z)-heptadeca-9(Z),12(Z),15(Z)-octadecatrienoic acid. The identity of the hydroperoxide with 2(R)-hydroperoxy-9(Z),12(Z),15(Z)-octadecatetraenoic acid was based on chemical and spectral analyses as described under “Results”.

Methods for Estimation of the Stability of 2-Hydroperoxides—The rate of breakdown of 2-hydroperoxides in enzyme preparations or in buffer was determined by using one of two methods. In method A, tritium-labeled 2(R)-hydroxy-9(Z),12(Z),15(Z)-octadecatetraenoic acid (specific activity, 35 M youthful) was stirred at 23 °C with enzyme preparation or buffer (0.8 or 1 ml). At different times of stirring, the sample was subjected to RP-radio-HPLC using a column protected with a pre-column and a solvent system consisting of acetonitrile/water (80:20, v/v) at a flow rate of 2 ml/min. Remaining hydroperoxide was eluted as its salt (3.6–6.0 ml effluent), well separated from the main product of hydroperoxide breakdown, i.e. 8(Z),11(Z),14(Z)-heptadeca-9(Z),12(Z),15(Z)-octadecatrienoic acid. The presence of enzyme preparations, a portion of the product consisting of 2-hydroxy-9(Z),12(Z),15(Z)-octadecatetraenoic acid was determined with a Packard model 5907B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph fitted with a methylsilicone capillary column (100 m; film thickness, 0.33 µm) and a flame ionization detector. Helium at a flow rate of 25 cm/sec was used as the carrier gas. Retention times found on GLC were converted into radiochemical purity using one of two methods. In method I (less than 1% of the recovered product), 22.9 ml effluent), 2 (1%), and compound 3 (34%) (Fig. 2). As with method A, no attempt was made to correct for the small amount of 2-hydroxy acid produced from the hydroperoxide during the incubation period. In some experiments, the reduced samples containing methyl 2-hydroxyoleate were derivatized with (−)-menthoxy-carbonyl chloride, purified by TLC, and subjected to GC-MS operated in the selected ion monitoring mode using the ions m/z 294 and 262. By comparing the peaks of the MC derivatives of methyl 2-hydroxyoleate (retention time, 3.6 min) and methyl 2(R)-hydroxyoleate (13.37 min) with the half-life data, it was possible to separately monitor breakdown of the “R” and “S” enantiomers of 2-hydroxyoleate.

Chemical Methods—Configuration determination of 2-hydroxy acids were performed by analysis of MC derivatives by GLC or GC-MS (30). MO derivatives of carbonyl compounds and Me3Si ethers of hydroxy compounds were prepared as described previously (31). [3H]Me3Si derivatives, occasionally needed to verify correct interpretation of mass spectra, were prepared by derivatization with [3H]Me3SiCl. Isotopic enrichment of the methyl ester of compound 1 by oxidation of linolenic acid with 2(R)-hydroxy-9(Z),12(Z),15(Z)-octadecatetraenoic acid was determined with a Packard model 1650 Fourier transform-infrared spectrophotometer. Radioactivity was determined with a Packard Tri-Carb model 4450 liquid scintillation counter (Packard Instruments, Downer’s Grove, IL).

RESULTS

Oxidation of Fatty Acids by Recombinant Oxigenases

Incubation of Linolenic Acid with Recombinant Enzymes from Tobacco Leaves or Arabidopsis—A preparation of the tobacco leaf oxygenase obtained from insect cells infected with baculovirus carrying pFASTBAC-tob.A5.2 was stirred for 20 min at 23 °C with 100 µM [9,10,12,13,15,16-3H]linolenic acid. The product isolated by extraction with diethyl ether (recovery of radioactivity, 87%) was analyzed by RP-radio-HPLC. Three peaks of radioactive compounds appeared in addition to the peak of linolenic acid remaining unconverted (Fig. 1A). Peaks 1 (7% of the recovered product, 22.9 ml effluent), 2 (1%, 38.4 ml effluent), and 3 (29%, 85.8 ml effluent) were collected for structural determination. As seen in Fig. 1B, a corresponding incubation using cells infected with virus carrying the pFASTBAC vector only gave undetectable conversion of the added tritium-labeled linolenic acid. Incubation of preparations from cells infected with virus carrying pFASTBAC-ara.N38086 (Arabidopsis oxygenase) gave rise to the same products as those formed by the tobacco leaf enzyme, i.e. compound 1 (4%), compound 2 (1%), and compound 3 (34%) (Fig. 2A). As seen in Fig. 2B, the same products, and an additional one (compound 4) eluting just after compound 1, were produced upon incubation of linolenic acid with a preparation of cucumber (see below).

Identification of Compound 1—The UV spectrum of compound 1 was featureless, indicating the absence of conjugated double bonds. On RP-HPLC, compound 1 co-chromatographed with authentic 2-hydroxylinolenic acid. Considerable chromatographic tailing was observed for both compounds. The retention time found on GLC (C-value, 18.96) and the mass spectrum of the methyl ester of compound 1, were identical to those of methyl 2-hydroxylinolenic acid (see “Experimental Procedures”). Results obtained upon GC-MS analysis of the Me3Si derivatives of the methyl esters of compound 1 and 2-hydroxylinolenic acid were identical. Catalytic hydrogenation of compound 1 followed by esterification produced methyl 2-hydroxy-9(Z),12(Z),15(Z)-octadecatetraenoic acid. Analysis by GLC of the MC derivative of the methyl ester of compound 1 showed that the absolute configuration at both the C-2 and C-3 positions was R (less than 1% of the S enantiomer). Degradation of the MC derivative of the methyl ester of compound 1 by oxidative ozonolysis produced the MC derivative of 2(R)-hydroxy-9(Z),12(Z),15(Z)-octadecatetraenoic acid (2(R)-hydroxylinolenic acid) (Fig. 3).

Identification of Compound 2—Compound 2 co-chromato-
graphed with authentic $8(Z),11(Z),14(Z)$-heptadecatrienoic acid on RP-HPLC. Furthermore, the retention time found on GLC (C-value, 16.76) and the mass spectrum of the methyl ester were identical to those of methyl $8(Z),11(Z),14(Z)$-heptadecatrienoate. Catalytic hydrogenation of compound 2 followed by methyl-esterification afforded methyl heptadecanoate, whereas oxidative ozonolysis of compound 2 yielded suberic acid as the main non-volatile fragment. On the basis of these results, compound 2 was identified as $8(Z),11(Z),14(Z)$-heptadecatrienoic acid (nor-linolenic acid) (Fig. 3).

Identification of Compound 3—Compound 3 co-chromatographed with authentic $8(Z),11(Z),14(Z)$-heptadecatrienal on RP-HPLC. Both compounds gave an unusually broad but symmetric peak (cf. Figs. 1 and 2). The C-value found on GLC (15.78) and the mass spectrum were identical to those of $8(Z),11(Z),14(Z)$-heptadecatrienal. Furthermore, the MO derivatives of compound 3 and the authentic reference had identical C-values and mass spectra. Reduction of compound 3 with NaBH$_4$ afforded $8(Z),11(Z),14(Z)$-heptadecatrienol, the Me$_3$Si derivative of which gave a mass spectrum showing prominent ions at $m/z$ 322 (M$^+$; 3), 307 (M$^+$ − 15; loss of CH$_3$; 2), 266 (3), 183 (4), 135 (11), 108 (54), 79 (99), and 75 (100). Catalytic hydrogenation of reduced compound 3 afforded 1-heptadecanol. Based on these results, compound 3 was identified as $8(Z),11(Z),14(Z)$-heptadecatrienal (Fig. 3).

Products formed from Linoleic Acid—Preparations of the tobacco leaf and Arabidopsis enzymes (2 and 0.5 mg of protein, respectively) in 5 ml of potassium phosphate buffer, pH 7.4, were stirred at 23 °C for 30 min with 70 mM linoleic acid. The product was treated with diazomethane and analyzed by GLC and GC-MS. Apart from methyl linoleate (C-17.69) corresponding to unreacted linoleic acid, three peaks appeared having the following C-values: C-15.73, C-16.71, and C-18.96. The mass spectrum recorded on the first mentioned peak showed prominent ions at $m/z$ 250 (M$^+$; 4%), 221 (1), 207 (1), 193 (1), 179 (1), 151 (3), 95 (36), 81 (62), 67 (100), and 55 (61), thus indicating a C$_{17}$ diunsaturated aldehyde. Treatment with O-methylhydroxylamine afforded two peaks due to the syn/anti isomers of

![Fig. 1. Reversed phase HPLC radioch chromatograms of products formed from linolenic acid incubated with preparations from insect cells. Panel A, [9,10,12,13,15,16-$^3$H$_6$]linolenic acid (100 µM) was stirred with a preparation of cells expressing the tobacco leaf oxygenase in potassium phosphate buffer, pH 6.7 (5.5 ml; 2.4 mg of protein), at 23 °C for 20 min and the product was isolated by extraction with diethyl ether. Panel B, same as A but using a preparation of control cells not expressing oxygenase. Solvent system II at a flow rate of 1.5 ml/min was used. 1, 2, 3, compounds 1–3; 18:3, linoleic acid.](image1)

![Fig. 2. Reversed phase HPLC radiochromatograms of products formed from linolenic acid incubated with a preparation from insect cells or with a cucumber preparation. Panel A, [9,10,12,13,15,16-$^3$H$_6$]linolenic acid (100 µM) was stirred with a preparation of the Arabidopsis oxygenase in potassium phosphate buffer, pH 6.7 (10 ml, 1.1 mg of protein), at 23 °C for 20 min and the product was isolated by extraction with diethyl ether. Panel B, [9,10,12,13,15,16-$^3$H$_6$]linolenic acid (268 µM) was stirred with whole homogenate of cucumber at 23 °C for 20 min and the isolated product was analyzed by HPLC. Solvent system II at a flow rate of 1.5 ml/min was used. 1, 2, 3, 4, compounds 1–4; 18:3, linoleic acid.](image2)
the aldehyde, the mass spectra of which showed prominent ions at m/z 279 (M⁺; 1%), 264 (M⁺ – 15; loss of CH₃; 1), 248 (M⁺ – 31; loss of OCH₃; 57); 180 (11), 168 (7), 95 (30), 81 (57), 67 (100), and 55 (78). The second peak was due to methyl nor-linoleate as judged by its C-value (16.71) and its mass spectrum, which showed prominent ions at m/z 280 (M⁺; 12%), 249 (M⁺ – 31; loss of OCH₃; 7), 206 (4), 150 (16), 95 (52), 81 (81), 67 (100), and 55 (75). The last peak (C-18.96) provided a mass spectrum showing a molecular ion at m/z 310 in agreement with a 2-hydroxyoctadecadienoate methyl ester. A more informative spectrum was recorded on the Me₃Si derivative, i.e. m/z 382 (M⁺ + 2%), 367 (M⁺ – 15; loss of CH₃; 20), 323 (M⁺ – 59; loss of COOCH₃; 18), 306 (3), 233 (4), 161 (Me₅SiO⁺ = CH-COOCH₃; 4), 159 (14), 129 (Me₅SiO⁺ = CH-CH = CH₂; 14), 89 (Me₅SiO⁺; 43), and 73 (Me₅Si⁺; 100). On the basis of these data, the linoleic acid-derived compounds were identified as 8(Z),11(Z)-heptadecadienal, 8(Z),11(Z)-heptadecadienoic acid, and 2-hydroxy-8(Z),12(Z)-octadecadienoic acid (Table I). The same set of compounds was produced from linoleic acid (100 μM) upon incubation with a whole homogenate preparation of cucumber.

**Products Formed from Oleic Acid**—Incubation of 70 μM oleic acid with the tobacco leaf or Arabidopsis enzyme preparations carried out as described for linoleic acid provided three compounds which were identified by their C-values and mass spectra as 8(Z)-heptadecenal, 8(Z)-heptadecenoic acid, and 2-hydroxy-8(Z)-octadecenoic acid (Table I). The mass spectrum of the aldehyde showed a molecular ion at m/z 252, which was shifted to m/z 281 upon treatment with O-methylhydroxylamine. The methyl ester of the 2-hydroxy acid produced a prominent ion at m/z 312 (4%) and a prominent ion at m/z 253 (24%) formed by elimination of the carboxemethoxy group. Incubation of oleic acid (100 μM) with the cucumber preparation resulted in the formation of an identical set of products.

**Products Formed from Palmitic Acid**—Palmitic acid (70 μM) incubated with the tobacco leaf, Arabidopsis, or cucumber enzyme preparations produced three compounds which were identified as pentadecanal, pentadecanoic acid, and 2-hydroxy-9(Z)-octadecenoic acid. The two first mentioned compounds gave C-values and mass spectra which were identical to those of the authentic compounds, and the 2-hydroxy acid gave identical data as those of the 2-hydroxy acid formed upon stannous chloride reduction of 2-hydroperoxymalmitic acid.

**Kinetic Constants of Recombinant Oxygenases**—Fatty acids were stirred with preparations of the tobacco leaf or Arabidopsis enzymes (protein, 0.13 and 0.07 mg/ml, respectively) at 30 °C in 1.5 ml of 0.1 M Tris buffer, pH 8.0, and the rate of oxygen uptake was monitored using a Clark oxygen electrode. The Kₘ and Vₘₐₓ values were determined from double-reciprocal plots of the maximum velocity of oxygen uptake and substrate concentration. The results are given in Table II. As seen, linolenic, linoleic, and oleic acids were all good substrates for the two enzymes and had Kₘ values of 1–2 μM (tobacco leaf oxygenase) and 13–18 μM (Arabidopsis oxygenase). In agreement with previous results (22), arachidonic acid was a less effective substrate compared with the 18-carbon fatty acids.

**Biosynthesis of 2(R)-Hydroperoxylinalenic Acid**

Formation of 2(R)-Hydroperoxylinalenic Acid by Recombinant Enzymes—Arabidopsis enzyme in 0.1 M potassium phosphate buffer, pH 7.4 (5 ml; protein, 0.2 mg/ml), was stirred at

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**Table I**

| Substrate    | Products                                      | C-value |
|--------------|-----------------------------------------------|---------|
| Linolenic    | 8(Z),11(Z),14(Z)-Heptadecatrienal             | 15.78   |
| Linoleic     | 8(Z),11(Z)-Heptadecadienal                    | 15.73   |
| Oleic        | 8(Z)-Heptadecenal                             | 15.79   |
| Palmitic     | Pentadecanal                                  | 14.01   |
|              | 2-Hydroxy-9(Z)-octadecenoic acid              | 16.79   |
|              | 2-Hydroxy-9(Z)-octadecenoic acid              | 19.00   |
|              | Pentadecanoic acid                            | 15.00   |

* C-values found on analysis by GLC were calculated from the retention times of underivatized aldehydes and of methyl esters of carboxylic acids.
0 °C for 20 min with 50 μM [9,10,12,13,15,16-3H6]linolenic
acid. The product was rapidly extracted with diethyl ether and
subjected to RP-radio-HPLC. As seen in Fig. 4, in addition to
compounds 1–3 (3, 2, and 8%, respectively, of the recovered
radioactivity), an additional peak of radioactivity appeared
immediately after compound 1. This material, i.e. compound 4
(24% of the recovered radioactivity), was converted into com-
 pound 1 (2-hydroxylinolenic acid) upon treatment with mild
 reducing agents such as SnCl2 or triphenylphosphine, suggest-
ing that it was due to the 2-hydroperoxy derivative of linolenic
acid. Reduction of compound 4 with sodium borodeuteride led
to the formation of 2-hydroxylinolenic acid with no detectable
incorporation of deuterium, thus excluding the presence of a
keto function. Analysis of compound 4 by GC-MS without de-
rivatization resulted in thermally induced decarboxylation and
the appearance of a single peak giving the same mass spectrum
as 8(Z),11(Z),14(Z)-heptadecatrienal. A similar analysis car-
ried out following trimethylsilylation using BSTFA reagent
showed peaks due to 8(Z),11(Z),14(Z)-heptadecatrienal (6.4
min retention time, 23%), the Me3Si ester of 2-ketolinolate
(10.3 min, 12%), the Me3Si ether/ester of 2-hydroxylinolate
(10.8 min, 17%), the Me3Si ether/ester of the enol form of
2-ketolinolate (11.0 min, 13%), and the Me3Si peroxide/ester
of 2-hydroperoxylinolate (11.5 min, 35%). This profile of
products was analogous to that observed for the Me3Si deriva-
tive of 2-hydroperoxylate (see “Experimental Procedures”).
The mass spectrum of the derivative of 2-hydroperoxylinole-
tenate showed prominent ions at m/z 439 (M+ – 15; loss of
CH3; 2%), 422 (M+ – 32; rearrangement with loss of CH2OH;
12), 337 (M+ – 117; loss of COO+SiMe3; 2), 247 (337–90; 8), 163
(Me3SiO-O” = SiMe3; 18), 147 (Me3SiO” = SiMe2; 39), 89
(Me3SiO”; 52), and 73 (Me3Si; 100) (Fig. 5A). This fragmen-
tation was analogous to that observed for the Me3Si derivative
of 2-hydroperoxylate. In another experiment, the Arabidop-
sis enzyme was incubated with linolenic acid under an atmos-
phere of 18O2. An aliquot of compound 4 isolated by RP-HPLC
was treated with BSTFA and subjected to GC-MS analysis.
Analysis of the isotope composition using the ions formed by
elimination of CH3 (m/z 439, 441, and 443) showed the pres-
ence of molecules containing 2 atoms of 18O (78%), 1 of 18O
(4%), and no 18O (18%). Although the extent of 18O labeling
was not complete, it was clear that compound 4 was formed from
linolenic acid by incorporation of one molecule of dioxygen, as
would be expected for a hydroperoxide. The mass spectrum of
the Me3Si derivative of 18O2-labeled compound 4 showed the
expected shifts compared with the spectrum of the unlabeled
derivative, i.e. (number of 18O in parentheses): 443 (2), 424 (1),

**TABLE II**

**Kinetic constants of recombinant oxygenases**

| Substrate       | pFASTBAC-tob.A5.2 | pFASTBAC-ara.N38086 |
|-----------------|-------------------|---------------------|
|                 | Km (μM) | Vmax (nmol O2/min/mg) | Km (μM) | Vmax (nmol O2/min/mg) |
| Linolenic acid  | 2       | 46                  | 18      | 119                  |
| Linoleic acid   | 2       | 33                  | 17      | 111                  |
| Oleic acid      | 1       | 24                  | 13      | 91                   |
| Palmitic acid   | 8       | 32                  | 11      | 46                   |
| Arachidonic acid| 8       | 32                  | 11      | 46                   |

**Fig. 4.** Reversed phase HPLC radiochromatogram of product formed from linolenic acid incubated with a preparation from
insect cells at 0 °C. [9,10,12,13,15,16-3H6]Linolenic acid (50 μM) was stirred with a preparation of cells expressing the Arabidopsis oxygenase at
0 °C for 20 min and the product was isolated by extraction with diethyl ether. Solvent system I (0–25 min) followed by system IV (25–40 min) at
a flow rate of 1.8 ml/min was used. 1, 2, 3, 4, compounds 1–4; 18:3, linolenic acid. The peak of UV absorption due to BHT present in the diethyl
ether used has been substracted.

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**TABLE II**

**Kinetic constants of recombinant oxygenases**

Consumption of O2 by protein extracts containing recombinant oxygenases from tobacco (pFASTBAC-tob.A5.2) or Arabidopsis (pFASTBAC-ara.N38086) was evaluated after incubation with increasing concentrations of various fatty acids in 0.1 M Tris buffer, pH 8.0, at 30 °C. Kinetic constants were estimated from Lineweaver-Burk plots.
On the basis of the data described, compound 4 was identified as 2-hydroperoxy-9(\textit{Z}),12(\textit{Z}),15(\textit{Z})-octadecatrienoic acid (2-hydroperoxylinolenic acid). The absolute configuration at C-2 was \textit{R} as shown by GLC analysis of the MC derivative of 2-hydroxylinolenic acid prepared by reduction with SnCl\textsubscript{2}.

Incubation of Linolenic Acid with Enzyme Preparations from Cucumber—[9,10,12,13,15,16-\textsuperscript{3}H\textsubscript{6}]Linolenic acid (268 \textmuM) was stirred with whole homogenate of cucumber at 23 °C for 20 min and the product isolated by extraction with diethyl ether (recovery of radioactivity, 74%) was analyzed by RP-radio-HPLC. As seen in Fig. 2B, the main products formed from the labeled linolenic acid were compound 1 (10\% of the recovered radioactivity), compound 2 (3\%), compound 3 (18\%), and compound 4 (8\%). Compounds 1–4 were identified as described above and found to be 2(\textit{R})-hydroxylinolenic acid, 8(\textit{Z}),11(\textit{Z}),14(\textit{Z})-heptadecatrienoic acid, and 2(\textit{R})-hydroperoxylinolenic acid, respectively. The radioactive materials eluting before compound 1, and the materials eluting between compounds 4 and 2, were lipoxygenase products as judged by the finding that lipoxygenase inhibitors blocked their formation. The fraction sedimenting at 48,000 \textit{g} of homogenate of cucumber was a convenient source of dioxygenase activity. By incubating linolenic acid with this fraction at 0 °C (see Fig. 6 and “Experimental Procedures”) it was possible to prepare milligram amounts of 2(\textit{R})-hydroperoxylinolenic acid.

### Conversions of 2-Hydroperoxy Fatty Acids

#### Degradation of 2(\textit{R})-Hydroperoxylinolenic Acid in Buffer—

Tritium-labeled 2(\textit{R})-hydroperoxylinolenic acid (35 \textmuM) was added to 0.1 M potassium phosphate buffer, pH 7.4, and kept at 23 °C for 30 min. The product was analyzed by RP-radio-HPLC and found to consist of unchanged hydroperoxide (48\%), 8(\textit{Z}),11(\textit{Z}),14(\textit{Z})-heptadecatrienial (46\%), and 8(\textit{Z}),11(\textit{Z}),14(\textit{Z})-heptadecatrienoic acid (6\%). Longer times of treatment led to further loss of hydroperoxide with concomitantly increased formation of heptadecatrienial and heptadecatrienoic acid. An increased ratio of heptadecatrienoic acid/heptadecatrienial was observed with longer times of treatment, indicating that the heptadecatrienial acid was formed from the aldehyde by air oxidation. Analysis of hydroperoxide degradation using method A demonstrated a first order decay with a rate constant of 0.0234 min\textsuperscript{-2} corresponding to a half-life time of 30 min. 2-Hydroxylinolenic acid was not detectable in these experiments.

#### Degradation of 2(\textit{R})-Hydroperoxylinolenic Acid in Enzyme Preparations—

Tritium-labeled 2(\textit{R})-hydroperoxylinolenic acid (25 \textmuM) was added to preparations of cells expressing the tobacco leaf or \textit{Arabidopsis} enzymes, or none of these enzymes, and kept at 23 °C for 20 min. Analysis by RP-radio-HPLC showed that only trace amounts of hydroperoxide remained. The product compositions were similar in the three incubations and consisted of 8(\textit{Z}),11(\textit{Z}),14(\textit{Z})-heptadecatrienial (about 90\%), 2-hydroxy-9(\textit{Z}),12(\textit{Z}),15(\textit{Z})-octadecatrienoic acid (about 7\%), and 8(\textit{Z}),11(\textit{Z}),14(\textit{Z})-heptadecatrienoic acid (about 3\%). Degradation of the hydroperoxide followed first-order kinetics with \( k = 0.221–0.245 \text{ min}^{-1} \) corresponding to half-life times ranging from 2.8 to 3.1 min. No significant difference in the rates of degradation of hydroperoxide in the presence of tobacco leaf or \textit{Arabidopsis} enzymes or in their absence was noticeable (Fig. 7).
Degradation of 2(R,S)-Hydroperoxyoleic Acid in Enzyme Preparations—Tritium-labeled 2(R,S)-hydroperoxyoleic acid (30 μM) was stirred at 23 °C with preparations of recombinant oxygenases or with the 48,000 × g particle fraction of homogenate of cucumber. Analysis by RP-radio-HPLC demonstrated a rapid degradation of the hydroperoxide with concomitant formation of 8(Z)-heptadecenal, 2-hydroxy-9(Z)-octadecenoic acid, and 8(Z)-heptadecenoic acid. The rate of hydroperoxide degradation as determined by method B followed first-order kinetics with half-life times of 5.9 min (cucumber preparation) or 2.4–2.7 min (preparations of insect cells expressing the tobacco leaf or Arabidopsis enzymes). In order to determine whether degradation of the hydroperoxide was associated with chiral discrimination, samples removed at the different time points were derivatized with (2)-menthoxycarbonyl chloride and subjected to steric analysis. As shown in Fig. 8, no significant difference in the rates of degradation of the 2(R)- and 2(S)-enantiomers of the hydroperoxide was noticeable (half-life times for 2(R)- and 2(S)-enantiomers in the presence of tobacco leaf or Arabidopsis enzymes, 2.7 and 2.5 min, respectively; half-life times for 2(R)- and 2(S)-enantiomers in the presence of Arabidopsis enzyme, 2.4 and 2.5 min, respectively). Similar results were obtained in incubations with preparations of cells not expressing oxygenases.

2(R)-Hydroperoxylinolenic Acid as a Substrate for Peroxygenase—A particulate fraction from homogenate of V. faba seeds containing peroxygenase (6) was stirred with 5,8,11,14-eicosatetraynoic acid (50 μM) at 23 °C for 5 min in order to block lipoxygenase activity and subsequently treated with [9,10-3H2]oleic acid (100 μM) and 2-hydroperoxylinolenic acid at 23 °C for 15 min. Control incubations performed in the absence of 2-hydroperoxylinolenic acid, or in the presence of 9(S)-HPOD, were also performed. Conversion of the tritium-labeled oleic acid into cis-9,10-epoxyoctadecanoic acid (6) was monitored by RP-radio-HPLC. As seen in Fig. 9, 2-hydroperoxylinolenic acid supported epoxidation of oleic acid into 9,10-epoxyoctadecanoic acid.
Hydroperoxyoleic acid (30 μM) was added to enzyme preparations (6.5 ml) and kept at 23 °C. Aliquots (1.2 ml) were removed during the time interval 1–10 min and added to ethanol containing stannous chloride. Amounts of 2-hydroxyoleate, reflecting residual 2-hydroperoxyoleate, were determined by GLC (method B). Samples were derivatized with (−)-menthoxycarbonyl chloride and the enantio-meric composition of 2-hydroxyoleate at the different time points was determined by GC-MS. A, incubation with tobacco leaf peroxynasase; B, incubation with Arabidop-sis oxydases. ■, 2(R,S)-hydroperoxyxyleate; ●, 2(R)-hydroperoxyxyleate; ○, 2(S)-hydroperoxyxyleate.

In another experiment, 2-hydroperoxyoleic acid (17 μM) was subjected to TLC using a solvent system of ethyl acetate:hexane, 20:80 (v/v). Two bands appeared, the less polar of which ($R_p = 0.59$) was due to methyl 2-hydroxy-9(Z)-octadecenoate as judged by GC-MS analysis. The more polar material ($R_p = 0.32$) was analyzed as its Me$_3$Si derivative by GC-MS. Prominent ions were observed at $m/z$ 369 (M$^+$ − 31; loss of OCH$_3$; 28), 341 (M$^+$ − 59; loss of COOCH$_3$; 15), 325 (34), 251 (341−90; 4), 199 (12), 159 (18), 129 (Me$_3$SiO$^+$ = CH−CH = CH$_2$; 18), and 73 (Me$_3$Si$^+$; 100). Treatment of the material with perichloric acid in aqueous tetrahydrofuran afforded methyl 2,9,10-trihydroxyoctadecenoate, thus confirming the identity of the compound produced from 2-hydroperoxyxyleic acid in the presence of peroxynasase as 9,10-epoxy-2-hydroxyoctadecenoic acid.

**DISCUSSION**

A pathogen-inducible oxygenase (PIOX) in tobacco leaves and a homologous enzyme from Arabidopsis were identified in recent work (22). Sequence and functional analysis of the piox cDNA-encoded protein showed significant homology with prostaglandin-endoperoxide H synthase types-1 and -2, key enzymes in the synthesis of lipid signal molecules in vertebrates. Endoperoxide synthases are dual function enzymes possessing cyclooxygenase and peroxidase activities (34). The recombinant PIOX proteins from tobacco and Arabidopsis possessed oxygenase activity toward several polyunsaturated fatty acids, however, peroxidase activity could not be demonstrated (22).

In order to establish the catalytic function of the oxygenase from tobacco leaves and its homologue from Arabidopsis, incubations with linolenic acid were performed and the isolated products were characterized by chemical and spectral methods. With both enzymes, the major compound consisted of a C$_{17}$ unsaturated aldehyde, which was identified as 8(Z),11(Z),14(Z)-heptadecatrienal by comparison with authentic material. In addition, small amounts of 2(R)-hydroxylinolenic acid and the C$_{17}$ homologue of linolenic acid, i.e. 8(Z),11(Z),14(Z)-heptadecatetraenoic acid, were produced. Other fatty acids, including linoleic, oleic, and palmitic acids, were metabolized in an analogous way (Tables I and II). The product profile observed, consisting of a chain-shortened aldehyde, a 2-hydroxy acid, and a chain-shortened fatty acid, was the same as the profile encountered previously in studies of α-oxidation in plant tissues. This metabolic pathway was characterized by Stumpf (35), who found that a preparation from peanut cotyledons catalyzed the oxidation of palmitic acid into a long chain fatty aldehyde with concomitant liberation of CO$_2$. In subsequent work, α-oxidation of various C$_n$ fatty acids into C$_{n-1}$ aldehydes together with varying amounts of C$_n$-hydroxy acids and C$_{n-1}$ fatty acids has been studied in preparations of pea leaves (36), cucumber (27–29), potato (37), and the green alga Ulva pertusa (38). The

**FIG. 9. Peroxynasen-catalyzed epoxidations of oleic acid.** Suspensions of the 105,000 × g particle fraction of homogenate of V. faba (0.5 ml) were stirred with 50 μl 5,8,11,14-eicosatetraynoic acid at 23 °C for 5 min and subsequently treated with 100 μl tritium-labeled oleic acid and hydroperoxide at 23 °C for 15 min. Formation of tritium-labeled 9,10-epoxyoctadecanoic acid was monitored by RP-radio-HPLC. ●, incubations with 2(R)-hydroperoxylinolenic acid; ○, incubations with 9(S)-HPOD.
α-Dioxygenases

α-oxidation enzymes have been suggested to operate together with aldehyde dehydrogenase and NAD⁺ and thus to provide a pathway for stepwise degradation of fatty acids into shorter chain homologues (for review, see Ref. 39).

When the recombinant enzymes from tobacco and Arabidopsis were incubated with substrate at 0 °C rather than at room temperature, formation of aldehyde was suppressed and a new main product was formed, i.e. compound 4 (Fig. 4). Compound 4 was converted into 2(R)-hydroperoxylinolenic acid upon treatment with mild chemical reductants and underwent thermal decarboxylation into 8(Z),11(Z),14(Z)-heptadecatrienial. These results indicated that compound 4 was identical to 2(R)-hydroperoxylinolenic acid, a new member of the oxylipin family of compounds. The trimethylsilyl peroxide/ester derivative of 2-hydroperoxylinolenic acid was sufficiently stable to be analyzed by gas chromatography-mass spectrometry (Fig. 5A; cf. Refs. 40 and 41). As expected, an incubation carried out under 15O gas resulted in incorporation of 15O₂ and formation of doubly 15O-labeled hydroperoxide (Fig. 5B). Isolation of 2(R)-hydroperoxylinolenic as the main product of oxygenation of linolenic acid defined the tobacco and Arabidopsis enzymes as fatty acid α-dioxygenases (Fig. 10). The product profile observed following incubation of linolenic acid with the well studied α-oxidation system in cucumber (27–29) (Figs. 2B and 6) was similar to that observed in the corresponding incubations with the recombinant α-dioxygenases (Figs. 1A, 2A, and 4), thus suggesting the general involvement of α-dioxygenase in plant α-oxidation.

Isolation and characterization of 2(R)-hydroperoxylinolenic acid was of interest in relation to the mechanism of α-oxidation. Already in 1974, Shine and Stumpf (42) found that inclusion of glutathione and glutathione peroxidase to incubations of palmitic acid with systems for α-oxidation resulted in increased formation of 2-hydroxymalmitic acid and a concomitant decrease in the formation of aldehyde and CO₂. On the basis of this result, a 2-hydroperoxide was proposed as an intermediate in α-oxidation (42). This hypothesis was recently supported by the finding that incubations of fatty acids with a preparation from pea leaves carried out in the presence of stannous chloride afforded enantiomerically pure 2(R)-hydroxy acids at the expense of aldehydes (43), and very recently by the isolation of 2(R)-hydroperoxymalmitic acid in incubations of palmitic acid with the α-oxidation system from the green alga U. pertusa (44).

2(R)-Hydroperoxylinolenic acid isolated in the present work was considerably less stable than the lipoxynegase-type of fatty acid hydroperoxides. The products formed upon nonenzymatic decomposition consisted of heptadecatrienial (about 90%) accompanied by small and variable amounts of 2-hydroxylinolenic acid and heptadecatrienial acid. Methodology for estimation of the rate of breakdown of 2-hydroperoxylinolenic acid was devised. Chemical degradation of the hydroperoxide in aqueous buffer, pH 7.4, at 23 °C followed first-order kinetics with a half-life time of about 30 min. The rate of decomposition was increased about 10-fold in the presence of preparations of cells expressing the recombinant α-dioxygenases. The proximal and distal heme-binding histidines of prostaglandin-endoperoxide H synthase-1 (Hsp300 and Hsp207, respectively) as well as the distal glutamine (Gln203) (34, 45) are conserved in the α-dioxygenases from tobacco and Arabidopsis (22), thus indicating that these enzymes are heme proteins capable of further transformation of fatty acid hydroperoxides. However, no specific hydroperoxide degrading activity could be detected for the recombinant α-dioxygenases, since the increased rate of hydroperoxide degradation observed with preparations of cells expressing α-dioxygenases was observed also with control cells not expressing α-dioxygenase (Fig. 7). Furthermore, degradation of a racemic hydroperoxide, i.e. 2(R,S)-hydroxyxyloleic acid, in the presence of α-dioxygenases proceeded in a non-stereoselective way (Fig. 8). Although it is conceivable that α-dioxygenases, when tested in purified form (cf. Ref. 29), will promote degradation of 2-hydroperoxides, the available data suggest that other tissue-derived factors will prove more important in this respect.

The α-oxidation pathway in mammals is of critical importance for degradation of phytic acid and other β-methyl branched fatty acids (46), however, the function of the corresponding pathway in plants is not fully understood. The fact that PIOX, now established as a fatty acid α-dioxygenase involved in α-oxidation, is pathogen-inducible, suggests that the importance of the α-oxidation pathway in plants may be related to plant-pathogen interactions and defense reactions rather than to serve as a pathway for stepwise degradation of fatty acids. Possibly, the 2-hydroperoxides generated by action of α-dioxygenases can act as signaling compounds for inductions of genes and enzymes of importance for plant’s defense against pathogens (cf. Ref. 47). A direct toxic effect of the hydroperoxide or its degradation products on the invading pathogen is also conceivable. Finally, because 2-hydroperoxides support peroxxygenase-catalyzed epoxidation (Fig. 9), biosynthesis of fungal toxic fatty acid epoxides (48) may take place by coupling of the α-dioxygenase and peroxxygenase pathways.

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