Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 11849–11853, December 1995
Plant Biology

Lipoxygenase-catalyzed oxygenation of storage lipids is implicated in lipid mobilization during germination

(*Cucumis sativus* L./hydroxy linoleic acid/lipid body/oxygenated lipids)

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Communicated by J. Schell, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany, September 5, 1995

ABSTRACT **The etiolated germination process of oilseed plants is characterized by the mobilization of storage lipids, which serve as a major carbon source for the seedling. We found that during early stages of germination in cucumber, a lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) form is induced that is capable of oxygenating the esterified fatty acids located in the lipid-storage organelles, the so-called lipid bodies. Large amounts of esterified (13*S*)-hydroxy-(9*Z*,11*E*)-octadecadienoic acid were detected in the lipid bodies, whereas only traces of other oxygenated fatty acid isomers were found. This specific product pattern confirms the *in vivo* action of this lipoxygenase form during germination. Lipid fractionation studies of lipid bodies indicated the presence of lipoxygenase products both in the storage triacylglycerols and, to a higher extent, in the phospholipids surrounding the lipid stores as a monolayer. The degree of oxygenation of the storage lipids increased drastically during the time course of germination. We show that oxygenated fatty acids are preferentially cleaved from the lipid bodies and are subsequently released into the cytoplasm. We suggest that they may serve as substrate for β-oxidation. These data suggest that during the etiolated germination, a lipoxygenase initiates the mobilization of storage lipids. The possible mechanisms of this implication are discussed.**

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) catalyze the regio- and stereoselective insertion of molecular oxygen into a (1*Z*,4*Z*)-pentadiene system of polyunsaturated fatty acids, forming hydroperoxy fatty acids (1). They have been identified in plants and in various animal tissues (2, 3). Historically, lipoxygenases have been classified according to their positional specificity of arachidonic acid oxygenation. 5-Lipoxygenase introduces molecular oxygen at carbon atom 5 (C-5) of arachidonic acid, whereas 12- and 15-lipoxygenases oxygenate this substrate at C-12 and C-15, respectively. In higher plants arachidonic acid is either not found or is a very minor constituent; thus, an arachidonic acid-related nomenclature may be misleading. For a more comprehensive classification of lipoxygenases, other enzymatic properties should be applied (4). Despite our knowledge of the protein chemistry, molecular biology, and enzymology of plant lipoxygenases (1, 3), there is no general concept for their biological function. Plant lipoxygenases have been implicated in leaf senescence in plant growth and development, in the response to wounding and pathogen attack, and in nitrogen partitioning (for review, see refs. 1 and 5). Large amounts of lipoxygenases have been detected in the seeds of various plants (6), but it still remains unclear whether there is a function for these enzymes during the germination process.

To investigate the biological role of a lipoxygenase, knowledge of its subcellular localization and its endogenous substrates is of major importance. In soybean and cucumber, particulate and soluble lipoxygenase forms have been described in various parts of the plants (4, 7–13). During the germination of cucumber seeds, a lipoxygenase form with an unusual alkaline pH optimum was detected at the membranes of the lipid bodies (14). Recently, we showed that this enzyme is activated when it binds to the lipid body membrane (15). Since the storage lipids serve as a major carbon source for the seedling during germination in the dark, the binding of a lipoxygenase at the lipid bodies and its activation suggest that this enzyme may have a specific function during the germination process.

We investigated the *in vivo* action of the lipid body lipoxygenase and found that the enzyme oxygenates the storage lipids containing linoleic acid (9,12-octadecadienoic acid) residues to their (13*S*)-hydro(pero)xy derivatives. The lipoxygenase reaction is paralleled by a specific release of the oxidized fatty acids from the lipid bodies into the cytosol. These data suggest that this specific lipoxygenase is implicated in lipid mobilization during the germination process.

EXPERIMENTAL PROCEDURES

Materials. Standards of chiral and racemic hydroxy fatty acids were from Cayman Chemicals (Ann Arbor, MI), and methanol, hexane, 2-propanol (all HPLC grade) were from Baker. Cucumber (*Cucumis sativus* L. cv. Chinesische Schlange), soybean (*Glycine max* cv. Maple Arrow), tobacco (*Nicotiana tabacum* cv. Samsun), and rape (*Brassica napus* L. cv. Lirawell) plants were used.

Plant Growth and Isolation of Lipid Bodies. Cotyledons of dry seeds or of seedlings germinated for the indicated times in the dark were used for experiments. The lipid bodies were prepared by ultracentrifugation (14). For each experiment, lipid bodies were prepared from 20 cotyledons.

Lipid Extraction and Sample Workup. The isolated lipid bodies were resuspended in 1 ml of 0.1 M phosphate buffer (pH 7.4) and sonicated to achieve homogenous dispersion. The sample was acidified to pH 3 with 1 M HCl, and the neutral lipids were twice extracted with 2 ml of hexane. The organic phase was recovered, the solvent was evaporated, and the lipids were redissolved in 0.1 ml of chloroform. To obtain the phospholipids, the water phase of the hexane extracts was reextracted with a 2:1 (vol/vol) mixture of chloroform/methanol (16). After removal of the organic phase, the solvents were evaporated, and the lipids were reconstituted in 0.1 ml of chloroform. Aliquots (80 μl) of both hexane and chloroform extracts were subjected to reversed-phase-HPLC (RP-HPLC) for quantification of free hydroxy fatty acid derivatives. The remaining extracts were diluted with 0.4 ml of

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Abbreviations: CP-HPLC, chiral-phase HPLC; RP-HPLC, reversed-phase HPLC; SP-HPLC, straight-phase HPLC; 13-HPODE, (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid; 13-HODE, (13*S*)-hydroxy-(9*Z*,11*E*)-octadecadienoic acid.

methanol, 80 μl of 40% (wt/vol) KOH was added, and the samples were hydrolyzed under argon atmosphere for 20 min at 60°C. After cooling down to room temperature, the samples were acidified with glacial acetic acid, and aliquots were subjected to RP-HPLC for determination of the degree of oxygenation of the lipid-body ester lipids. For the determination of the free fatty acid content in the cytosol, the supernatant of the first ultracentrifugation step (see above) at 200,000 × *g* was acidified to pH 3 and twice extracted with 1 ml of ethyl acetate. After recovery of the organic phase, the solvents were evaporated and the lipids were reconstituted in 0.1 ml of methanol.

Analytics. HPLC analysis was carried out on a Shimadzu HPLC system coupled to a diode array detector. RP-HPLC was performed on a Nucleosil C_{18} column (Macherey & Nagel, KS-system; 250 × 4 mm, 5-μm particle size). A solvent system of methanol/water/acetic acid [85/15/0.1 (vol/vol)] and a flow rate of 1 ml/min was used. The absorbances at 235 nm (detection of the conjugated diene system of the hydroxy fatty acids) and at 210 nm (detection of the nonoxygenated polyenoic fatty acids) were recorded. Compounds separated in the chromatograms were quantified by peak areas. Calibration curves (five point measurements) for (13*S*)-hydroxylinoleic acid and linoleic acid were established. Straight-phase HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (DuPont; 250 × 4.6 mm, 5-μm particle size) with a solvent system of *n*-hexane/2-propanol/acetic acid [100/2/0.1 (vol/vol)] and a flow rate of 1 ml/min. The enantiomer composition of the hydroxy fatty acids was analyzed by chiral-phase HPLC (CP-HPLC) on a Chiralcel OD column (Diacel Chemicals, J. T. Baker; 250 × 4.6 mm, 5-μm particle size) with a solvent system of hexane/2-propanol/acetic acid [100/5/0.1 (vol/vol)] and a flow rate of 1 ml/min. Compounds were generally identified by coinjections with authentic standards.

Gas chromatography/mass spectrometry (GC/MS) was performed with a Hewlett–Packard GC/MS system equipped with a capillary RSL-150 column (polydimethyl siloxane, 0.25-mm coating thickness, 30 m × 0.32 mm; Research Separation Laboratories, Eke, Belgium). The hydroxy fatty acid prepared by SP-HPLC was methylated with diazomethane, repurified on SP-HPLC, silylated with bis-(trimethylsilyl)trifluoroacetamide in the presence of dry pyridine, and then subjected to GC/MS analysis. For more informative mass spectra, catalytic hydrogenation of the hydroxy fatty acid methyl esters was carried out.

RESULTS

Lipoxygenase Products Occur in Lipid Bodies of Germinating Cucumber Cotyledons. Immunohistochemical studies revealed that a novel lipoxygenase isoform is induced at early stages of germination of cucumber seeds and that this enzyme is found to be associated with the membrane of the lipid-storing organelles (12, 17). To find out whether the enzyme oxygenates the storage lipids *in vivo*, we analyzed the lipid extracts for the occurrence of specific lipoxygenase products. Fig. 1 shows a representative RP-HPLC chromatogram of the hydrolyzed lipid extracts (total lipid extract as described in ref. 16) from lipid bodies isolated on day 3 of germination. Products comigrating with authentic standards of (13*S*)-hydroxy-(9*Z*,11*E*)-octadecadienoic acid (13-HODE) were detected when the chromatogram was recorded at 235 nm. These compound(s) were characterized by a conjugated diene chromophore (Fig. 1 *Inset A*). Recording the chromatogram at 210 nm (Fig. 1, lower trace), we analyzed the polyenoic fatty acid composition of the lipid bodies. It can be seen that linoleic acid is the major polyenoic fatty acid in the lipid bodies (>90%). Quantification of the chromatographic traces at 235 nm and at 210 nm allowed the calculation of the hydroxylinoleic acid/linoleic acid ratio, which appears to be a suitable measure for the degree of oxidation of the storage lipids. On day 3 of germination, this value varied between 0.07 and 0.13, indicating that roughly 1 of 10 linoleic acid molecules was present as the oxygenated derivative. For more detailed structural information, the compound(s) absorbing at 235 nm was prepared by RP-HPLC and further analyzed by SP- and CP-HPLC. In SP-HPLC (not shown), one major product comigrating with an

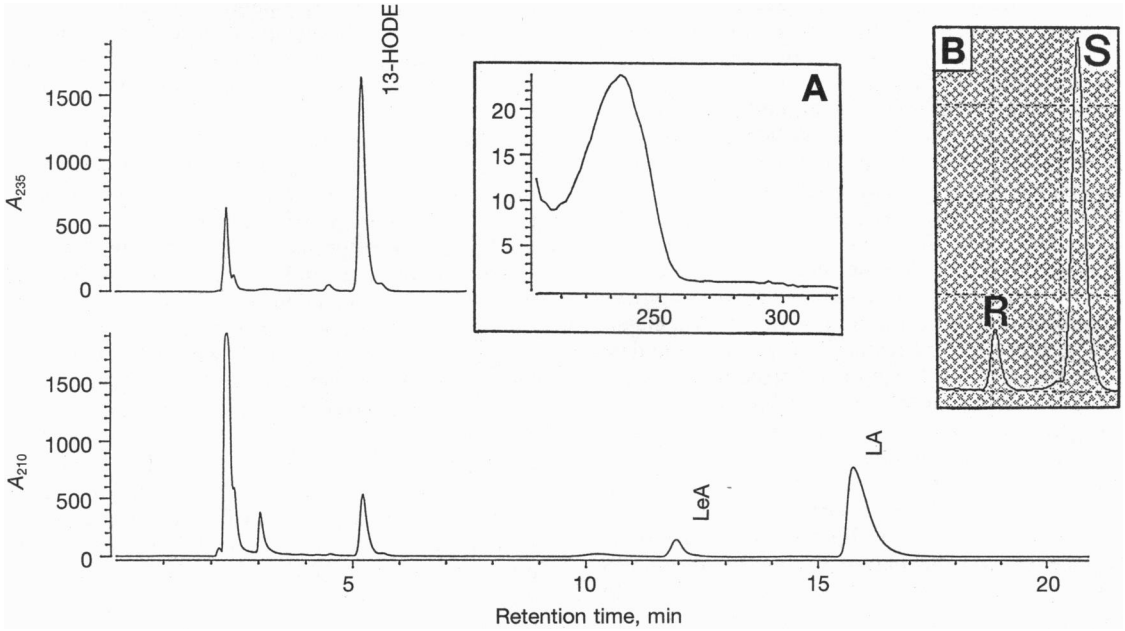

FIG. 1. HPLC analysis of hydroxy fatty acids from the lipid bodies of germinated cucumber seedlings. Cucumber seeds were germinated for 72 hr in the dark. The lipid bodies were prepared, the lipids were extracted, and the extracts were hydrolyzed under alkaline conditions. The resulting free fatty acid derivatives were analyzed by RP-HPLC as described in text. The absorbances at 235 nm (detection of the conjugated diene system of the hydroxy fatty acids) and 210 nm (detection of the nonoxygenated polyenoic fatty acids) were recorded. (*Inset A*) UV spectrum of the compound(s) eluted at 5.4 min in SP-HPLC. (*Inset B*) CP-HPLC (analysis of the enantiomer composition) of 13-HODE prepared by SP-HPLC. LA, linoleic acid; LeA, linolenic acid.

authentic standard of 13-HODE was detected. GC/MS analysis (data not shown) of the methylated trimethylsilyl ether of this compound provided independent evidence of its chemical structure. Analysis of the enantiomer composition (CP-HPLC) indicated a preponderance of the *S* isomer (Fig. 1 *Inset B*). In the nonhydrolyzed lipid extracts, no free 13-HODE was detected, indicating a location of the hydroxy fatty acids in the ester lipid fraction. The specific formation of esterified 13-HODE during germination may be due to the action of the lipid-body lipoxygenase. Interestingly, the lipid extracts did not contain any hydroperoxy fatty acids, which are the primary products of the lipoxygenase reaction. Since hydroperoxy lipids were shown to survive at least in part in our workup procedure (18), a rapid *in vivo* reduction of the compounds to their stable hydroxy derivatives may be assumed.

Degree of Oxidation of the Storage Lipids Increases During Germination. If the lipoxygenase-catalyzed oxidation of storage lipids is related to the process of germination, a time-dependent increase in the degree of oxygenation of the storage lipids may be expected during the time course of germination. As a suitable measure for the degree of oxygenation, we determined the hydroxylinoleic acid/linoleic acid ratio. From Fig. 2*A* it can be seen that in the dry seeds at 0 hr, small amounts of hydroxy fatty acids were detected. SP-HPLC indicated an almost equal distribution of (9*R*)- and (13*S*)-hydroxy-(9*Z*,11*E*)-octadecadienoic acid and 13-HODE (not shown), and CP-HPLC (Fig. 2*B*) revealed a racemic mixture (*S*/*R* ratio of 1). These data suggest that lipoxygenase may not be involved in the biosynthesis of these products. At later stages of germination the hydroxylinoleic acid/linoleic acid ratio increased. This increase is mainly due to the *in vivo* action of the lipoxygenase as indicated by the increasing *S*/*R* ratio (Fig. 2*B*).

The neutral storage lipids are surrounded by a monolayer of phospholipids (19, 20). To oxidize the neutral lipids, the lipoxygenase has to penetrate through the phospholipid monolayer. In other words, the acyl groups of the phospholipids should be the primary targets for oxygenation. We separated the phospholipids from the neutral lipids by a sequential extraction procedure and analyzed the hydroxylinoleic acid/linoleic acid ratio in both extracts (Fig. 2*A*). Hydroxy fatty acids were detected in both lipid subfractions but not in the fraction of free polyenoic fatty acids. During the time course of germination, the hydroxylinoleic acid/linoleic acid ratio was always higher in the phospholipids than in the neutral lipids; at 96 hr of germination, that ratio in the phospholipid compartment was 0.55, indicating that more hydroxylinoleic acid than linoleic acid was present. Such a high degree of oxidation is expected to hamper severely the structure of the phospholipid monolayer and thus may initiate the decomposition of the lipid bodies.

Hydroxy Fatty Acids Are Preferentially Released from the Lipid Bodies. The utilization of the fatty acids housed in the lipid bodies as carbon source requires their liberation from the ester lipids and their subsequent degradation via β-oxidation (21, 22). To test whether the lipoxygenase-catalyzed oxidation of the storage lipids has any impact on the liberation of fatty acids from the lipid stores, we analyzed the free fatty acid pattern in the cytosol of cucumber seedlings during the time course of germination. From Fig. 3 it can be seen that only small amounts of free linoleic acid and its (13*S*)-hydroxy derivative (13-HODE) were present in the dry seeds at 0 hr. During the investigated time course, the amount of linoleic acid was below the detection limit of 0.1 μg per sample. To exclude mistakes, we indicate the detection limit as the amount found for linoleic acid. During germination there was a drastic increase in the cytosolic concentration of 13-HODE. In contrast, we did not detect any time-dependent change in the linoleic acid concentration. These data may be interpreted as a result of a preferential release of hydroxylinoleic acid from the lipid bodies. However, the cytoplasmic steady-state concentrations of both hydroxylinoleic acid and linoleic acid depend on their liberation from the lipid bodies and on their secondary metabolization via β-oxidation. Thus, the low cytosolic concentration of linoleic acid may be due to its subsequent catabolism. To exclude this possibility, we prepared lipid bodies from dry cucumber seeds, and from 96-hr-old cucumber seedlings, incubated them for 2 hr in phosphate buffer (pH 8.0), and quantified the amount of free hydroxylinoleic acid and linoleic acid released into the medium. We found that only small amounts of hydroxylinoleic acid (0.03 μg) were liberated from the lipid bodies prepared from dry cucumber seeds. In contrast, the lipid bodies of 96-hr-old cucumber seedlings released much more 13-HODE (1.1 μg). On the other hand, we did not detect any free linoleic acid in the incubation buffer. These data suggest a preferential hydrolysis and release of hydroxylinoleic acid from the lipids of this organelle because we could not detect any free hydroxylinoleic acid in the lipid bodies and because a secondary catabolism of linoleic acid derivatives in the incubation buffer is unlikely.

Oxygenation of Storage Lipids Also Occurs in Other Plants. To determine whether the oxygenation of storage lipids during

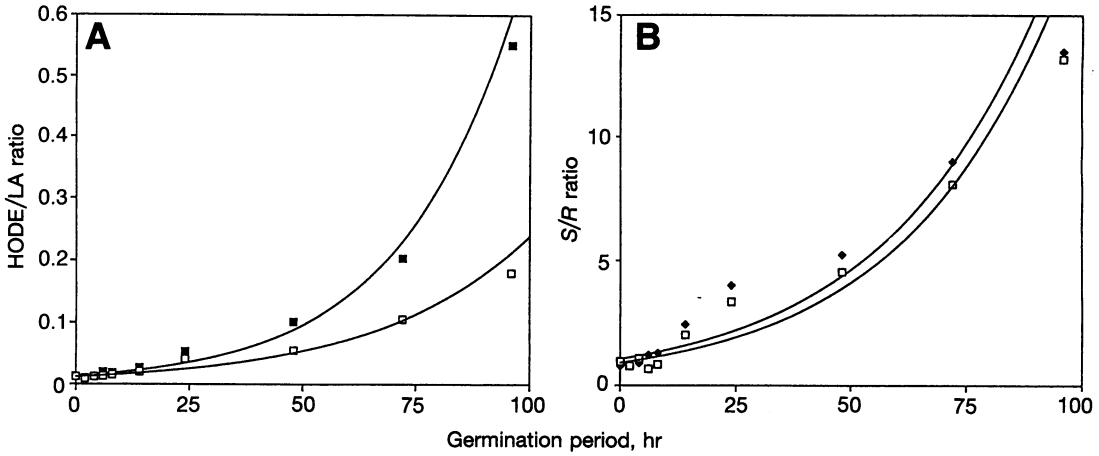

FIG. 2. Formation of lipoxygenase products during the time course of germination. Cucumber seeds were germinated in the dark for various time periods, and the lipid bodies were prepared from the cotyledons. The neutral lipids (□) were extracted with hexane; subsequently, the phospholipids (■ in A, ◆ in B) were recovered as described (16). After alkaline hydrolysis, the hydroxylinoleic acid/linoleic acid ratio (HODE/LA ratio) was determined by RP-HPLC (*A*). The hydroxy fatty acid fraction was recovered from RP-HPLC, the 13-HODE was separated from the traces of other hydroxy linoleic acid isomers by SP-HPLC, and its enantiomer composition (*S*/*R* ratio) was determined by CP-HPLC (*B*).

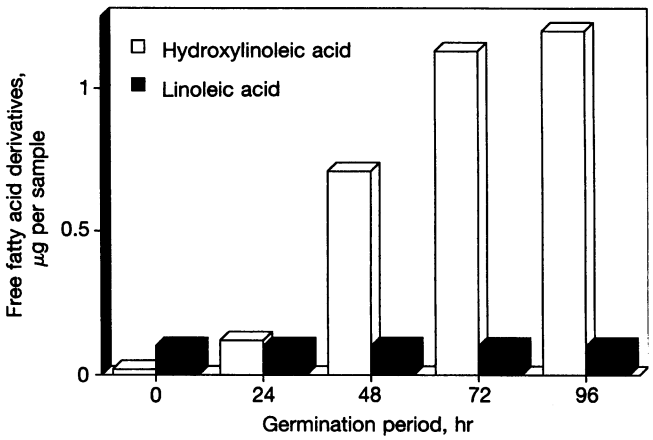

FIG. 3. Levels of free 13-HODE in the cytosol during the time course of germination. Cucumber seeds were germinated in the dark for various time periods, and the cytosol of the cotyledons (200,000 × *g* supernatant) was prepared by ultracentrifugation. After acidification to pH 3.0, the free fatty acid derivatives were extracted twice with equal volumes of ethyl acetate. The solvent was evaporated, and the remaining lipids were reconstituted in methanol and used for RP-HPLC analysis. The amount of free 13-HODE was quantified by peak area. The amount of free linoleic acid was below the detection limit (<0.1 μg per sample). To exclude mistakes, we indicate the detection limit as the amount found for linoleic acid.

early stages of germination is restricted to cucumber, we analyzed the lipid bodies of other oilseeds for the occurrence of oxygenated fatty acids. Large amounts of hydroxy fatty acids were detected in the lipid bodies of soybean, tobacco, and rape seedlings after 96 hr of germination (Table 1). In contrast to cucumber, tobacco and rape contained only small amounts of hydroxy fatty acids in dry seeds. We found large amounts of 13-HODE in dry soybean seeds. CP-HPLC analysis indicated an *S/R* ratio of 83/17, suggesting the involvement of a lipoxygenase. It is suggested that the lipoxygenase-catalyzed oxidation of the storage lipids proceeds already during seed development. Interestingly, in these three plants we detected no hydroperoxy derivatives of linoleic acid in lipid bodies, either.

DISCUSSION

Supply of energy from the metabolization of endogenous storage products is a crucial step during the germination process of plants. Some plants such as potato utilize storage starch for ATP production, whereas others, the so-called oilseeds, utilize storage lipids (21, 22). The mobilization of the storage lipids, which are located in the lipid bodies, is a major event in seedling growth (19, 20, 23). Although it is known that β-oxidation is involved in energy supply, the mobilization of the storage lipids has not been investigated in detail. It is of particular interest how the storage ester lipids are cleaved to yield free fatty acids that may be further metabolized via β-oxidation. In maize seedling scutella (23, 24), a special lipase has been described that is capable of cleaving the storage lipids. However, the enzymatic activity of a similar enzyme was near the detection limit in germinating cucumber seedlings (25). Here, we provide experimental evidence that the lipid body lipoxygenase, which is induced during early stages of germination, may be involved in this process. Since it is located at the membrane of the lipid-storage organelles, it may exhibit a dual function. (*i*) It catalyzes oxygenation of the polyunsaturated fatty acids of the phospholipid monolayer surrounding the storage triacylglycerols and thereby may disrupt the structure of the phospholipid monolayer. Thus, the storage lipids may become accessible to cytosolic enzymes. A similar process has been proposed for the degradation of mitochondria during maturation of erythrocytes (26). (*ii*) After disruption of the phospholipid monolayer, the lipoxygenase may penetrate to the storage lipids to oxygenate their linoleic acid residues. According to our data, hydroxylinoleic acid is preferentially cleaved from lipids by an as-yet-unidentified enzyme that leads to a higher steady-state concentration of 13-HODE in the cytosol (Fig. 3). Afterwards the 13-HODE may be utilized via β-oxidation steps, which differs from the β-oxidation of linoleic acid (27). The β-oxidation of linoleic acid involves the combined action of the acyl-CoA dehydrogenase and the 2,4-dienoyl-CoA reductase to convert the C12 = C13 cis double bond into a C11 = C12 trans double bond via the dehydratase pathway (27, 28). In the case of 13-HODE, this trans double bond is already present; thus, enoyl-CoA isomerase can act directly without the need for additional enzymes.

Table 1. Formation of hydroxy fatty acids during the germination of seeds of various plants

Plant	Hydroxylinoleic acid/linoleic acid ratio	
	Dry seeds (0 hr)	After 96 hr of germination
Tobacco	0.002	0.035
Rape	0.003	0.008
Soybean	0.055	0.028

Seeds of soybean, tobacco, and rape (1.3 g of each plant) were germinated for 96 hr. The lipid bodies were prepared from the dry seeds and from 96-hr-old seedlings, the lipids were extracted (16), the extracts were hydrolyzed under alkaline conditions, and the resulting free fatty acids derivatives were analyzed by RP-HPLC. The hydroxylinoleic acid/linoleic acid ratio was determined as a measure for the degree of oxidation of the storage lipids.

The *in vivo* action of the lipid body lipoxygenase during the time course of germination suggests a role of this enzyme in seedling growth. To prove this hypothesis, inhibitor studies and/or experiments with transgenic plants would be helpful. We tested various commercially available lipoxygenase inhibitors (phenylbutazon, nordihydroxyguaiaretinic acid, *n*-propylgallate, and ibuprofen) *in vitro* up to concentrations of 100 μM for their capability to inhibit the lipid-body lipoxygenase, but all of them turned out to be ineffective.

The observation that pure enantiomers of hydroxy fatty acids were not only detected in cucumber seedlings but also in other plants suggests that the oxygenation of storage lipids is not a species-specific process of this lipoxygenase form. One may assume that the formation of hydroxy fatty acids may serve as a signal for degradation of storage lipids during germination and thus may discriminate this process from "normal" lipid turnover. An enzyme that preferentially cleaves hydroxy fatty acids from the storage ester lipids has not been identified yet in cucumber. However, in *Ricinus* seeds, an enzyme has been detected that specifically cleaves hydroxyoleic acid (ricinolic acid) from ester lipids sources (29, 30). It may be speculated that a similar enzyme may be responsible for the preferential cleavage of hydroxylinoleic acid from the storage lipids in cucumber seedlings. The fact that hydroxy fatty acids were already detected in soybean seeds suggests that the time program of the synthesis of the lipid-body lipoxygenase and its subsequent action on the storage lipids is different in different plants. In soybean the lipoxygenase-catalyzed oxidation of storage lipids occurs already during seed development. These data agree with the findings of Vernooy-Gerritsen *et al.* (31), who detected a lipoxygenase at very early stages of germination (2 hr) and described a decline in alkaline lipoxygenase activity during germination.

Lipoxygenases convert polyenoic fatty acids to their corresponding hydroperoxy derivatives. We specifically looked for hydroperoxy lipids in cucumber lipid bodies but did not detect significant amounts. However, from model studies we know that hydroperoxy fatty acids survive at least in part the alkaline

hydrolysis (32). The most plausible explanation for these results would be an immediate *in vivo* reduction of the hydroperoxy lipids. In soybean and broad bean, peroxygenases have been identified that convert a hydroperoxy fatty acid in the presence of polyenoic fatty acids to the corresponding hydroxy compound and a fatty acid epoxide (33–35). It remains to be investigated whether such a peroxygenase is involved in the reduction of hydroperoxy lipids formed during the germination process.

The authors thank Dr. S. Rosahl for critically reading the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 363/B5 to I.F. and C.W.) and by a grant of the Deutsche Forschungsgemeinschaft to H.K. (Ku 961/1-1).

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