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The Homolytic and Heterolytic Fatty Acid Hydroperoxide Lyase-like Activities of Hematin

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Pentenols and pentene dimers are biosynthetized in plants by homolytic fatty acid hydroperoxide lyase (HPL) or HPL-like enzymes. It has been found that these compounds can modify the flavor of olive oil. Reactions between hematin and 13-hydroperoxy-octadecatrienoic acid resulted in the formation of the same compounds via a free radical reaction in which an alkoxyl radical derived from linolenic acid hydroperoxide undergoes a *β*-scission. (*Z*)-3-Hexenal has also been detected as a minor product of the reaction. It is bioconversed from the same substrate in plants by heterolytic HPL. Thanks to the redox cycle of its central iron, hematin has both homolytic and heterolytic HPL-like activities. © 2001 Academic Press

Key Words: **hematin; hydroperoxide lyase; linolenic acid hydroperoxides; 13-hydroperoxyoctadecatrienoic acid; pentenol; pentene dimer.**

In higher plants, mushrooms and algae, fatty acid hydroperoxide lyase (HPL) cleaves linoleic, and linolenic hydroperoxides into volatile compounds (aldehydes, alcohols, or hydrocarbons) and oxo-acids. Two categories of HPL have been reported, according to their cleavage type: Homolytic HPL cleaves the bond between a saturated carbon and the one bearing the hydroperoxide function (Fig. 1). The link between vinylic carbon and carbon bearing the hydroperoxide function is cleaved by heterolytic HPL (1). HPL is an enzyme involved in "the lipoxygenase pathway" in which lipoxygenase (LOX) peroxydizes linoleic and linolenic acids into their 9- and 13-hydroperoxide isomers (2). It has been demonstrated that HPL is a heme protein (3) belonging to the cytochrome P450 class (4)

Hematin is known for its ability to degrade fatty acid hydroperoxides (5). In free radical reactions, hematin catalysis leads to a wide range of nonvolatile degradation products among which epoxyalcohols, triols, and ketones with long carbon chains (6–8). No generation of volatile compounds catalysed by hematin has been reported.

In this study, we have shown for the first time that hematin has both the homolytic and heterolytic HPL-like activities when reacted with 13-hydroperoxy-(*Z*,*E*,*Z*)-9,11,15-octadecatrienoic acid (13-HPOTE). The analysis of the volatile products resulting from the breakdown of 13-HPOTE catalysed by hematin has revealed the same compounds as those biosynthetized by HPL or HPL-like enzymes in plants. The mechanisms of the reaction catalysed by hematin are also proposed.

MATERIALS AND METHODS

Materials. Linolenic acid, soybean lipoxygenase-1, and hemin were purchased from Sigma. 1-Heptanol was purchased from Fluka.

Synthesis of 13-HPOTE. 13-HPOTE has been synthesized according to (9) using commercial soybean lipoxygenase and linolenic acid. The hydroperoxides have been purified on a preparative C18 cartridge (from Waters, 5 g resin, 10 mm i.d.). Prior to purification, the pH of the reaction medium (50 ml) was adjusted to 3 with 6N hydrochloric acid. Hydroperoxides were applied on the C18 cartridge. They were washed with water (25 ml) and *n*-hexane (12 ml, in order to remove traces of residual lipids). The hydroperoxides were eluted with methanol.

Hydroperoxide concentration was determined by U.V. spectrometry at 234 nm taking the value of $\varepsilon = 25{,}000\ cm^{-1}\ M^{-1}$.

Spectrophotometric measurements. The kinetics of hydroperoxides and hematin degradations have been monitored using an Ultrospec 4000 UV-visible spectrophotometer from Pharmacia.

Hematin catalysed degradation of 13-HPOTE. Hematin in methanol/0.1 M NaOH (99:1 v/v) (1 mM, 1 ml) was added to 3.5 ml of 50 mM phosphate buffer (pH 7.7). Then 0.5 ml of 30 mM 13-HPOTE (in methanol) were added and the vial was immediately closed and incubated for 15 min at room temperature while being shaken. The products formed were extracted twice with 2 ml diethyl ether (containing 0.25 mM heptanol as an internal standard) and submitted to GC-FID and GC-MS analyses. For headspace analysis, the same

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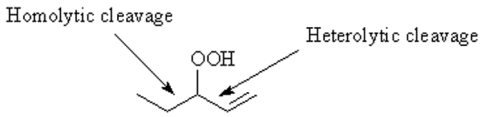

FIG. 1. Fatty acid hydroperoxides homolytic and heterolytic cleavages.

procedure was followed using a 10 ml headspace flask immediately sealed after addition of the hydroperoxides. After 2 h of stabilisation at 30°C, 500 μl of the headspace were sampled with a gas syringe and analyzed.

GC-FID and GC-MS analyse. A Hewlett-Packard HP6890 gas chromatograph fitted with a HP-5 MS column (28 × 0.25 mm, 1 μm film thickness) was used for both detection of pentene and pentene dimers in headspace. The temperature programme for pentene detection was the following: from 35°C (5 min) to 40°C at 1°C/min, then to 100°C at 5°C/min and finally to 250°C at 20°C/min. Better separation of pentene dimers was obtained with a CP-cyclodextrin column (Chrompack) of 24 × 0.25 mm, 0.25 μm film thickness and the following temperature program: 35 to 50°C (1°C/min), 180°C (10°C/min). The injector (splitless) and FID temperatures were maintained at 150 and 200°C, respectively. The linear velocity of the carrier gas (He) was maintained at 35 cm/s.

For analysis of ethereal extracts: 1 μl of ether extract was injected in a Hewlett-Packard HP5890 series II gas chromatograph coupled to a HP5972 mass spectrometer. A HP-5MS (30 × 0.25 mm, 1 μm film thickness) column was used with the following temperature program: from 35 to 125°C (5°C/min), then to 180°C (25°C/min).

RESULTS AND DISCUSSION

Hydroperoxide and Hematin Degradation

Reactions between 13-HPOTE and hematin resulted in decreases of both 234 nm (conjugated diene of 13-HPOTE) and 390 nm (hematin) absorbances (Fig. 2), which indicated that both 13-HPOTE and hematin were degraded during the reaction. An increase of the A280 has also been observed. This is caused by the conjugated oxodiene moiety of 13-oxotridecadienoic acid (13-OTA). The presence of this compound will be explained later in the discussion.

These observations are in line with those of Rafiquzzaman *et al.* (5) who have observed the same behaviour of methyllinoleate hydroperoxides in 98% methanol. Nevertheless, we have recorded a more that three times higher degradation rate of hematin in water than in methanol.

Identification of Volatile Metabolites

According to Degoussee *et al.* (10), pentane resulted from degradation of 13-HPODE. In an analogous reaction with 13-HPOTE, formation of pentene would be expected. However 13-HPOTE was degraded by hematin, no pentene was detected. Five unexpected peaks were systematically observed, sharing identical mass spectra (m/z, percentage relative intensity): 138 (<1%, molecular ion), 109 (17%), 95 (8%), 69 (68%), 68 (28%), 67 (25%), 41 (100%). They were identified as pentene dimers ($C_{10}H_{18}$). These pentene dimers have been characterised by Angerosa *et al.* (11) in virgin olive oil's aroma. Our recorded relative intensities of fragments fit exactly with those of these last authors. In olive oil's aroma, seven pentene dimers have been detected instead of five in our experiment. Using HP5-MS column, the last three dimers have the same retention times (Fig. 3) but using headspace sampling and a chiral column (see Materials and Methods), we succeeded in separating the seven pentene dimer molecules (data not shown).

Analysis of volatile compounds extracted by diethyl ether revealed two other less volatile products which

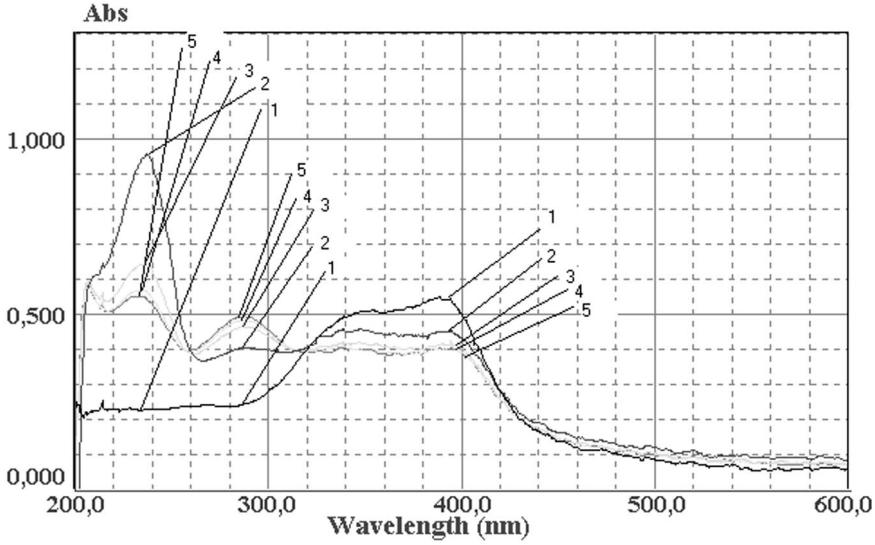

FIG. 2. UV/Vis spectra changes during the reaction between 0.013 mM hematin and 0.032 mM 13-HPOTE. 1, hematin spectrum; 2, adding 13-HPOTE; 3 to 5, scans 20, 40, and 60 s after the addition of hydroperoxides.

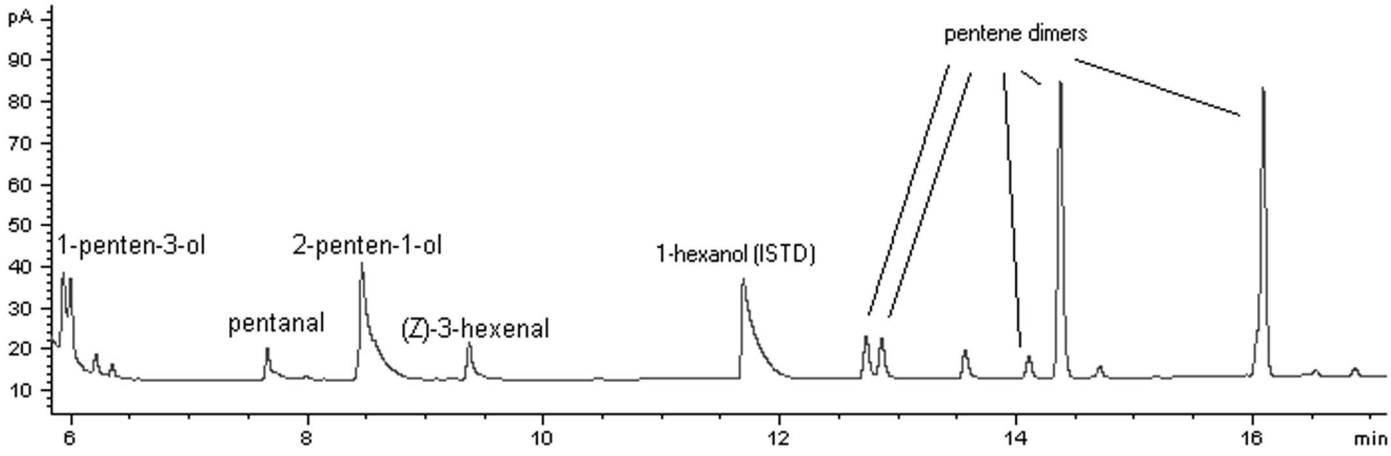

FIG. 3. GC-FID profile of volatile compounds resulting from incubation of 13-HPOTE with hematin.

were not recovered by headspace sampling: 1-Penten-3-ol and 2-penten-1-ol were identified with MS library matching and by coinjection with reference standards. Some additional minor compounds, such as pentenal and (*Z*)-3-hexenal, have also been identified by the same procedure. The relative distribution (area %) of the detected volatiles are presented in Table 1.

Biochemical Pathways Related to 13-HPOTE Degradation

This study showed for the first time that the hematin-catalysed 13-HPOTE breakdown produces nine major volatile compounds. The formation of pentenols and pentene dimers could be explained by a free radical mechanism detailed in Fig. 4: (a) The first step is the generation of an alkoxyl radical. (b) This radical undergoes a β-scission to give 13-oxo-tridecadienoic acid (13-OTA) and a pentenyl radical. The pentenyl radical may follow two rearrangement pathways: in the first (route 1), the radical would take the hydroxyl group back from hematin. Thanks to resonance, the withdrawn hydroxyl group may attach to C1 or C3 to give, respectively, 2-penten-1-ol or 1-penten-3-ol. In the second (route 2), two pentenyl radicals may react together giving one of the seven isomers of pentene dimers. The hydroxyl group remaining linked to hematin would lead to its degradation as proposed by White *et al.* (12).

TABLE 1

Distribution ($n = 3$, mean $\pm$ SD) of Volatile Compounds Generated by Degradation of 13-HPOTE by Hematin

Compounds	Molar yields
Pentenols[1]	41% ± 7%
Pentenal	5.6% ± 0.7%
Z-3-hexenals	4.4% ± 0.3%
Pentene dimers[2]	50% ± 14%

Note. 1, sum of 1-penten-3-ol and 2-penten-1-ol; 2, sum of the seven pentene dimers.

The results presented in Table 1 are in agreement with the recent observations made by Nunez *et al.* (13) who have detected the formation of pentene dimers, pentenols, and (*Z*)-3-hexenal formations after an incubation of 13-HPOTE with a purified enzyme from *Chlorella pyrenoidosa.* The real nature of this last enzyme remains unclear: It was first identified as an homolytic HPL. The recent results of Nunez *et al.* (13) have modified this conclusion and suggest that the enzymatic cleavage in *Chlorella* was due to an HPL-like activity of LOX in anaerobical conditions (13). Identical phenomenons have been observed both in virgin olive oils (11) and in soybean (14, 15). The exact identification of the enzyme responsible for the reaction remains also difficult: homolytic HPL or HPL-like activity of LOX. Another fact increased the complexity of this problem: If (according to the majority of the authors) LOX is responsible for the cleavage, the identification of the isoenzyme remains a total mystery. Indeed, multiple LOX activities have been described in every vegetable in which pentenols and pentene dimers have been encountered. In olive oils 9- and 13-hydroperoxides of both linoleic and linolenic acids can be formed (16); in *Chlorella,* a LOX that produces 9-, 10-, and 13-hydroperoxides isomers from linoleic acid has been described by (17); in soybean, LOX-1 produces 9- and 13-hydroperoxides if pH < 9 (18).

Using hematin as a catalyst, we have demonstrated that 13-HPOTE can be the precursor of these volatile compounds in a pure nonenzymatic way. Concomitant to volatile generation, the occurrence of 13-OTA has been confirmed by the increase of the absorbance at 280 nm as shown in Fig. 2 and as previously described (11, 13–15). The generation of other conjugated oxo-diene containing compounds have already been ob-

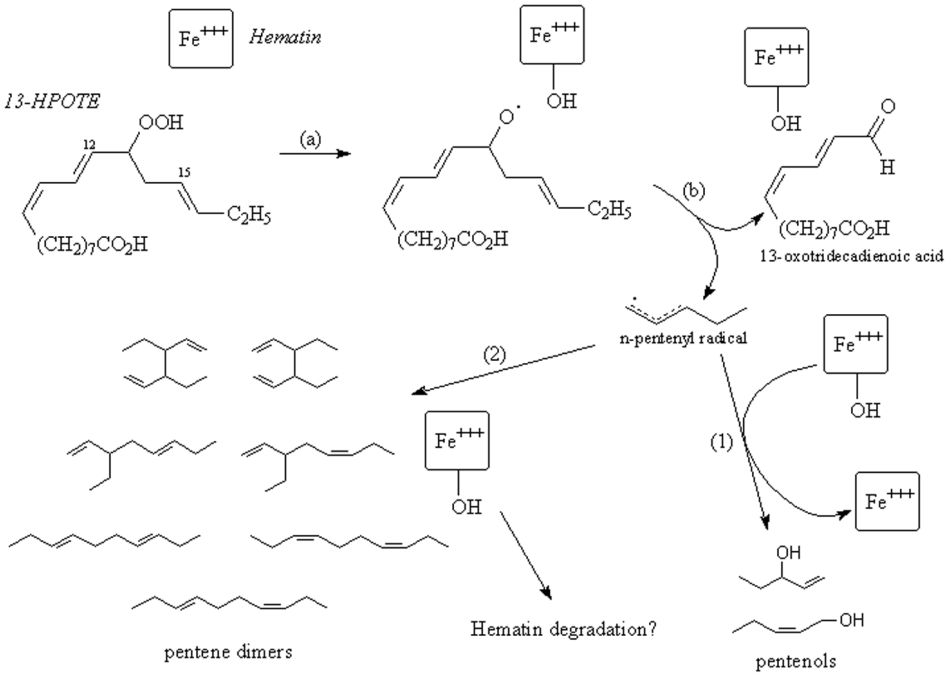

FIG. 4. Proposed scheme for the synthesis of pentenols and pentene dimers from 13-HPOTE breakdown catalysed by hematin.

served in analogous reactions in *Vicia sativa* and in rabbit leukocytes (19, 20).

In mushrooms, degradation of 10-hydroperoxyoctadecadienoic acid catalysed by homolytic HPL leading to 1-octen-3-ol has been reported (21). The reaction remains unclear but an analogous mechanism could be expected, a *n*-octenyl radical replacing *n*-pentenyl.

Hematin has also shown a minor heterolytic HPL-like activity: (*Z*)-3-hexenal has been systematically detected as a minor by-product in our experiments. The biosynthesis of this compound (also known as "leaf aldehyde") by heterolytic HPL has been reported for many plants (1). The weak levels of (*Z*)-3-hexenal could also be explained by a free radical mechanism as it was previously proposed for hexanal in (22): A radical intermediate compound is energetically defavorized by comparison with resonance stabilised n-pentenyl. Therefore, only relatively small quantities of (*Z*)-3-hexenal were detected in our experiments.

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