Detection of an enol intermediate in the hydroperoxide lyase chain cleavage reaction

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Abstract Guava (*Psidium guajava*) hydroperoxide lyase (HPL) preparations were incubated with [1-^{14}C](9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid for 1 min at 0°C, followed by rapid extraction/trimethylsilylation. Analysis of the trimethylsilylated products by gas chromatography–mass spectrometry and radio-high-performance liquid chromatography revealed a single predominant ^{14}C-labelled compound, identified by its ^{1}H-nuclear magnetic resonance, ultraviolet and mass spectra as the trimethylsilyl ether/ester of (9*Z*,11*E*)-12-hydroxy-9,11-dodecadienoic acid. Longer time incubations afford smaller yield of this enol due to its partial tautomerization into (9*Z*)-12-oxo-9-dodecenoic acid. The data obtained demonstrate that formation of (9*Z*)-12-oxo-9-dodecenoic acid in the HPL reaction is preceded by unstable enol oxylipin, and further suggest that hemiacetals are the true products of HPL catalysis.

Key words: Hydroperoxide lyase, CYP74B; Oxylipin; Enol; Mechanism; *Psidium guajava*

1. Introduction

The lipoxygenase pathway plays important roles in plant signalling and stress adaptation [1,2]. The primary lipoxygenase products, fatty acid hydroperoxides, are metabolized in higher plants by several enzymatic pathways. Molecular cloning of genes encoding allene oxide synthase [3], hydroperoxide lyase (HPL) [4], and divinyl ether synthase [5] revealed that these three groups of enzymes are all cytochrome P450 proteins belonging to a new CYP74 subfamily. All these enzymes are exceptionally catalytically active. For instance, the guava HPL performs about 2000 substrate turnovers per second [6].

Enzymes belonging to the HPL family catalyze cleavage of the carbon chain of lipoxygenase-derived fatty acid hydroperoxides resulting in the formation of an aldehyde and an ω-oxo-carboxylic acid [1,2]. The importance of this reaction in plant physiology and pathology stems from the fact that hexanal or (3*Z*)-hexenal produced by HPL action on linole(n)ic acid 13-hydroperoxides possess antimicrobial activity, and that the aldoacid counterpart, i.e. (9*Z*)-12-oxo-9-dodecenoic acid, is convertible by alkenal isomerase into traumatin, (10*E*)-12-oxo-10-dodecenoic acid, a plant wound hormone [7].

The mechanism of the hydroperoxide chain cleavage reaction catalyzed by HPL is not well understood. Studies using [$^{18}O_2$]13-hydroperoxides have shown incorporation of ^{18}O into the aldehyde group of the C_{12} cleavage product but not into the C_6 aldehyde [8], a result which has led to the proposal of an epoxyallylic cation (or radical) and a hemiacetal as intermediates [9–11]. To date no experimental proof for the existence of such intermediates has been presented, and the question whether the HPL reaction proceeds heterolytically or homolytically is unsolved.

The present paper is concerned with the detection of an enol oxylipin, (9*Z*,11*E*)-12-hydroxy-9,11-dodecadienoic acid, acting as an intermediate in the conversion of linolenic acid 13-hydroperoxide into (9*Z*)-12-oxo-9-dodecenoic acid catalyzed by HPL from guava (*Psidium guajava*).

2. Materials and methods

2.1. Materials

[1-^{14}C]Linolenic acid was obtained from DuPont NEN (Boston, MA, USA), and unlabelled linolenic acid was purchased from Nu-Chek-Prep (Elysian, MN, USA). [1-^{14}C]-labelled (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT; specific radioactivity 27.5 kBq/μmol) was prepared as described earlier [12]. Samples of (9*Z*)-12-oxo-9-dodecenoic and traumatin, (10*E*)-12-oxo-10-dodecenoic acid, were obtained from Larodan Chemical Co. (Malmö, Sweden).

Immature green fruits of guava (*P. guajava*) were bought in a local market in Stockholm. The central seed-containing part was removed and the remaining tissue was frozen and subsequently used for the incubations.

2.2. Enzyme preparation

Frozen fruit tissue (4 g) was ground with a grater. After addition of 8 ml of 100 mM phosphate buffer, pH 7.0, the resulting suspension was homogenized with Ultra-Turrax T25 (3×30 s at full speed, 0–4°C). The homogenate (pH about 4.0) was filtered through four layers of cheese-cloth and the filtrate (final volume 7–8 ml) used for the incubations. The procedure was scaled up five-fold for preparative experiments.

2.3. Analytical incubations and extraction

[1-^{14}C]13(*S*)-HPOT (400 μg; 35.5 kBq) in 50 μl of ethanol was added to filtrate prepared from 4 g of guava fruit at 0–4°C and the mixture was vortexed vigourously for 30–60 s. 3 ml of ice-cold pentane/diethyl ether (1:1, v/v) was added and the mixture was extensively shaken for a few seconds and then centrifuged for 30 s. The organic phase was quickly decanted and added to 1.5 ml of silylating mixture, i.e. pyridine/hexamethyldisilazane/trimethylchlorosilane 2:1:2 (v/v/v). The silylation was allowed to proceed for 30 min at ambient temperature. Solvent and silylation reagents were removed in vacuo and the remaining material was washed with 5 ml of hexane. The solvent was evaporated under a stream of argon and the dry residue was dissolved in 0.1 ml of hexane for gas chromatography–mass spectrometry (GC–MS) analyses.

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2.4. Preparative incubations and purification of trapping product

Preparative incubations were performed as described above but on a five-fold larger scale. The resulting products after silylation were purified by radio-high-performance liquid chromatography (HPLC) on a nitrile phase column Separon SIX CN (5 μm, 3.2×150 mm; Tessek, Prague, Czech Republic) with isocratic elution by the solvent mixture: hexane/diethyl ether 98:2 (v/v), flow rate 0.8 ml/min. Radioactivity detection was performed with an HPLC radiomonitor model 171 (Beckman Instruments, Fullerton, CA, USA) with a solid scintillator cell (125 μl).

2.5. Silylation of (9Z)-12-oxo-9-dodecenoic acid and (10E)-12-oxo-10-dodecenoic acid

Samples of (9*Z*)-12-oxo-9-dodecenoic acid and (10*E*)-12-oxo-10-dodecenoic acid (0.1 mg of each) were subjected to direct trimethylsilylation with 0.1 ml of silylation mixture. Work-up was performed as described above and the resulting products were analyzed by GC–MS.

2.6. Study of keto-enol tautomerism of (9Z)-12-oxo-9-dodecenoic acid in buffer or guava homogenate

(9*Z*)-12-Oxo-9-dodecenoic acid was dissolved in a small volume of ethanol and added to 2 ml of 100 mM phosphate buffer pH 6.0 (final concentration 165 μM) or to 5 ml of guava homogenate (final concentration 189 μM) at 0°C under vigorous vortexing. The mixtures were stirred at 0°C for 15 min and extracted with 0.3–0.4 volumes of cold pentane/diethyl ether 1:1 (v/v). The organic phase was decanted and transferred directly to 0.5 volumes of silylation mixture. The derivatized products were analyzed by GC–MS.

2.7. Spectral studies

UV spectra of purified products were recorded with a Hitachi U2000 instrument. Alternatively, during HPLC analyses UV topograms were recorded on line with an RSD 2140 diode array detector (LKB, Bromma, Sweden). Acquisition of spectral data was performed using Wavescan software (LKB, Bromma, Sweden). Products as TMS ester/ether derivatives were analyzed by GC–MS with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph equipped with a 5% phenylmethylsiloxane fused capillary column (length 12 m; film thickness 0.33 μm). Helium at a flow rate of 32 cm/s was used as the carrier gas. Injections were made in the split mode using an initial column temperature of 120°C. The temperature was raised at 10°C/min until 240°C. ^{1}H-nuclear magnetic resonance (NMR) spectra (400 MHz, [$^{2}H_6$]benzene, 298 K) were recorded with Bruker MSL-400 spectrometer.

3. Results and discussion

Short (30–60 s) incubations of guava homogenate with [1-^{14}C]13-HPOT at 0°C followed by rapid extraction with pentane/diethyl ether and immediate silylation of material present in the organic layer afforded a predominant silylated metabolite **I** (TMS/TMS-**I**) as revealed by GC–MS analysis (Fig. 1A). Control experiments were performed in which the fresh guava homogenate was replaced by boiled guava extract or buffer. GC–MS analysis of the silylated product obtained from such experiments showed the absence of TMS/TMS-**I** and the presence of the TMS ester of 13-oxo-9,11-tridecadienoic acid, a degradation product formed thermally from the TMS derivative of 13-HPOT during gas chromatography. These findings, coupled with the fact that the TMS ester of 13-oxo-9,11-tridecadienoic acid was undetectable in the guava incubations (Fig. 1A) demonstrated that (a) 13-HPOT was

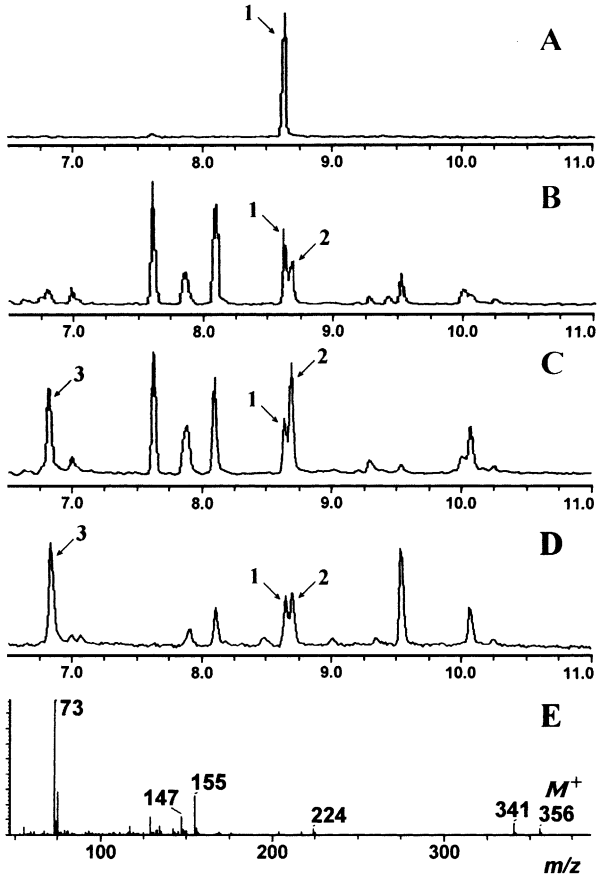

Fig. 1. GC–MS analyses of TMS derivatives of products formed in guava HPL incubations and control experiments. A–D: Total ion current chromatograms. A, products of 13-HPOT incubation (1 min, 0°C) with guava HPL preparation; B, similar, but 15 min incubation; C, (9*Z*)-12-oxo-9-dodecenoic acid incubation (15 min, 0°C) with guava HPL preparation; D, (9*Z*)-12-oxo-9-dodecenoic acid incubation (15 min, 0°C) in buffer (pH 6.0) without HPL; E, electron impact mass spectrum of compound **I** (TMS derivative). Peak 1 corresponds to TMS/TMS-**I**, peak 2 to the putative (11*Z*) isomer of TMS/TMS-**I**, peak 3 to TMS ester of (9*Z*)-12-oxo-9-dodecenoic acid.

Table 1
^{1}H-NMR spectrum of compound **I** TMS ether/ester (400 MHz, 298 K, $^{2}H_6$-benzene)

Protons	Chemical shift, δ (ppm)	Multiplicity (number of protons)	Coupling constants (Hz)
H12	6.65	d (1)	11.7 (H11)
H11	6.40	ddd (1)	11.3 (H10); −1.3 (H9)
H10	6.14	dd (1)	10.9 (H9)
H9	5.40	ddt (1)	7.5 (H8)
H8	1.90–2.10	m (2)	
H7	1.50–1.70	m (2)	
H6,5,4	1.32	m (6)	
H3	1.50–1.70	m (2)	
H2	2.35	t (2)	7.3 (H3)
TMS	0.28	s (9)	
TMS	0.06	s (9)	

virtually completely consumed during incubation with the guava preparation, and (b) compound **I** was an enzymatic product of 13-HPOT.

The electron impact mass spectrum of TMS/TMS-**I** (Fig. 1E) possessed the following most prominent ions *m*/*z* (ion attribution; relative intensity, %): 356 (M^+; 11), 341 (M^+-CH_3; 13), 224 (341−COOTMS; 8), 155 ($M^+-TMSOOC(CH_2)_6$; 36), 147 (18), 142 (6), 134 (9), 129 (16), 117 ($COOTMS^+$; 7), 75 (36), 73 (TMS^+, 100).

TMS/TMS-**I** was obtained in pure form following a large scale incubation and purification by normal phase HPLC. UV spectroscopy demonstrated an absorption band with $\lambda_{max} = 237$ nm (in hexane-diethyl ether 98:2 (v/v)) indicating the presence of two conjugated double bonds. Importantly, the absorption band was smooth, i.e. it lacked the two side bands present in the UV spectra of conjugated diene hydroperoxides and hydroxides [13] and was closely similar to that previously recorded on the allene oxide derivative (9*Z*)-12,13-epoxy-9,11-octadecadienoic acid [14]. The NMR data of TMS/TMS-**I** demonstrated the presence of a conjugated *Z*,*E*-diene system (Table 1, Fig. 2). The value of $J_{11,12}$ (11.7 Hz) indicated that the 12,13-double bond of TMS/TMS-**I** had the *E* configuration. This assignment followed *inter alia* from previous analyses of divinyl ether oxylipins by NMR, which had shown coupling constants of 11.5 Hz for *E* double bonds [15] and 6.2 Hz for *Z* double bonds [16] at the ether bridge.

The results obtained allowed us to identify TMS/TMS-**I** as the TMS ether/ester derivative of (9*Z*,11*E*)-12-hydroxy-9,11-dodecadienoic acid, i.e. the (11*E*) enol form of the aldehyde (9*Z*)-12-oxo-9-dodecenoic acid. It seemed possible that the enol might be produced secondary to the aldehyde by simple enolization rather than to act as the precursor of the aldehyde. The following experiments were carried out in order to distinguish between these two possibilities.

First, direct silylation of (9*Z*)-12-oxo-9-dodecenoic acid in the absence of aqueous buffer afforded a single component identified by GC–MS as the TMS ester of the (non-enolized) aldoacid (molecular ion, *m*/*z* 284). The TMS/TMS-**I** was undetectable. Secondly, when (9*Z*)-12-oxo-9-dodecenoic acid was incubated with buffer (pH 6.0) for 15 min at 0°C, analysis of the silylated product by GC–MS demonstrated a major peak due to the TMS derivative of the aldoacid (peak 3 in Fig. 1D). Two smaller peaks were also observed, the earlier eluting of which cochromatographed with the TMS/TMS-**I** (Fig. 1D, peaks 1 and 2). The mass spectra recorded on peaks 1 (TMS/TMS-**I**) and 2 were essentially identical, indicating that they were due to the 11(*E*) and 11(*Z*) isomers, respectively, of the TMS derivative of (9*Z*)-12-hydroxy-9,11-dodecadienoic acid. Thirdly, when 13-HPOT was incubated with the guava preparation for a longer time (15 min), peak 3 (TMS ester of aldoacid) as well as peaks 1 and 2 (TMS derivatives of 11(*E*)- and 11(*Z*)-isomers of enol) were observed upon GC–MS analysis (Fig. 1B). A similar result was obtained following incubation of (9*Z*)-12-oxo-9-dodecenoic acid with the guava preparation for 15 min, although there were quantitative differences in the product distribution (Fig. 1C). The relative proportion of enol isomers to aldoacid (peaks 1+2 relative to peak 3) was unexpectedly high in the control incubations performed (Fig. 1B–D); apparently the slightly acidic pH (4–6) shifted the equilibrium between aldehyde and enol forms towards the enol. The control experiments showed that (9*Z*)-12-oxo-9-dodecenoic acid when added to buffer or to the guava preparation, or was generated from 13-HPOT in the guava preparation, existed as an equilibrium mixture consisting of aldoacid and the 11(*Z*) and 11(*E*) forms of the corresponding enol form (Fig. 1B–D). This pattern of products was distinct from that observed following short incubation of 13-HPOT with the guava preparation, in which a single peak due to the TMS derivative of (9*Z*,11*E*)-12-hydroxy-9,11-dodecadienoic acid was observed (Fig. 1A). It followed that the enol detected in the short incubation did not have its origin in (9*Z*)-12-oxo-9-dodecenoic acid but was directly generated from 13-HPOT.

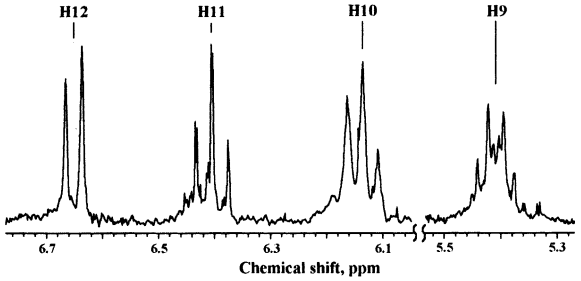

Fig. 2. Fragment of ^{1}H-NMR spectrum (400 MHz, [$^2H_6$]benzene, 298 K) of compound **I** TMS ether/ester, region of olefinic protons.

Detection of the enol (9*Z*,11*E*)-12-hydroxy-9,11-dodecenoic acid as a product formed from 13-HPOT in the presence of HPL throws light on the mechanism of the HPL-catalyzed chain cleavage reaction. From a chemical point, a logical precursor of the enol detected is a hemiacetal, and it seems likely that such a compound is the true product in HPL catalysis (Fig. 3; also cf. [9–11]). Apparently, the rate of cleavage of the putative hemiacetal into enol and (3*Z*)-hexenal is considerably more rapid than the rate of the conversion of enol into (9*Z*)-12-oxo-9-dodecenoic acid. In this context it should be pointed out that the enol detected in the present work should be

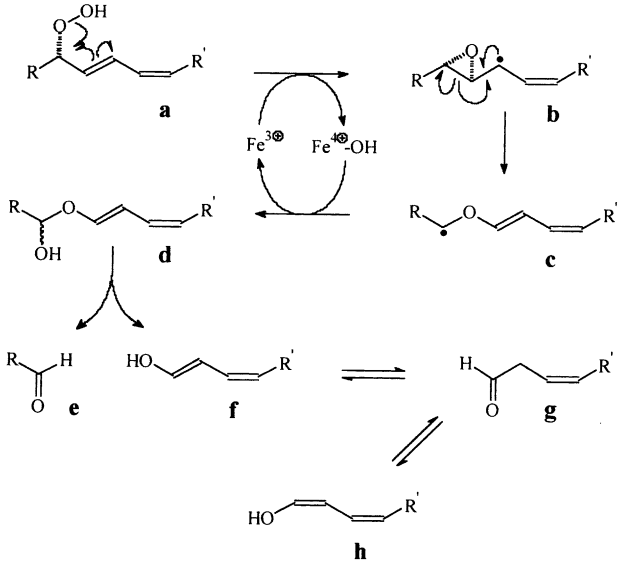

Fig. 3. Mechanism proposed for the HPL reaction. R = (2*Z*)-pentenyl; R′ = -$(CH_2)_7$COOH; a, 13-HPOT; b, epoxyallylic radical; c, vinyl ether radical; d, hemiacetal, (9*Z*,11*E*,3′*Z*)-12-(1′-hydroxy-3′-hexenyloxy)-9,11-dodecadienoic acid (compound **I**); e, (3*Z*)-hexenal; f, enol, (9*Z*,11*E*)-12-hydroxy-9,11-dodecadienoic acid; g, (9*Z*)-12-oxo-9-dodecenoic acid; h, enol isomer, (9*Z*,11*Z*)-12-hydroxy-9,11-dodecadienoic acid.

stabilized thermodynamically by the Δ^9 double bond which is in conjugation with the vinyl alcohol double bond. Further experiments directed to the detection of hemiacetal are under development in our laboratories.

It is well-known that HPL activity and HPL gene expression are induced by tissue wounding, infection and other stress conditions [17,18]. These conditions will lead to a sharply increased formation of the enol detected in the present study, and it cannot be excluded that the enol per se is relevant also from a physiological point.

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