The lipoxygenase pathway in garlic (*Allium sativum* L.) bulbs: detection of the novel divinyl ether oxylipins

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Abstract Incubations of [1-^{14}C]linoleic acid or [1-^{14}C]-(9*Z*,11*E*, 13*S*)-13-hydropero xy-9,11-octadecadienoic acid (13-HPOD) with juice of garlic bulbs lead to the formation of one predominant labelled product, viz., the novel divinyl ether (9*Z*,11*E*, 1′*E*)-12-(1′-hexenyloxy)-9,11-dodecadienoic acid ('etheroleic acid'). With lesser efficiency [1-^{14}C]α-linolenic acid or [1-^{14}C](9*Z*,11*E*, 13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT) are converted in this way into (9*Z*,11*E*,1′*E*,3′*Z*)-12-(1′,3′-hexadienyloxy)-9,11-dodecadienoic acid ('etherolenic acid'). Thus, garlic bulbs possess the activity of a new 13-hydroperoxide-specific divinyl ether synthase.

Key words: Linoleic acid; α-Linolenic acid, Lipoxygenase; 3- Hydroperoxides metabolism; Divinyl ether oxylipin; Garlic; *Allium sativum*

1. Introduction

Lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) is a widespread enzyme in plant tissues. The major substrates of lipoxygenases in plants are linoleic and α-linolenic acids. Most of lipoxygenases possess high stereo- and regio-specificity, producing 13(*S*)- or 9(*S*)-hydroperoxides. There are a few known plant enzymes, controlling the secondary conversions of hydroperoxides [1,2].

Conversion of (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadi enoic (9-HPOD) and (9*S*,10*E*,12*Z*,15*Z*)-hydroperoxy-10, 12,15-octadecatrienoic (9-HPOT) acids into the divinyl ethers colneleic (I) and colnelenic (II) acids, respectively (see Fig. 1) is one of the non-common routes of the plant lipoxygenase pathway. This route was discovered by Galliard and his colleagues in potato tubers [3–6]. Divinyl ether synthase from potato utilises specifically 9-HPOD and 9-HPOT. This enzyme is not efficient with 13-hydroperoxides as substrates. Until now, synthesis of divinyl ethers remained to be the unique property of potato tubers; it was not found in any other plant or animal species.

The present paper is concerned with detection of a new divinyl ether synthase in garlic (*Allium sativum*) bulbs. This enzyme uses preferentially 13-hydroperoxides (especially 13-HPOD) as substrates and efficiently converts them into the novel divinyl ether fatty acids.

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Abbreviations: 13-HPOD, (9*Z*,11*E*,13*S*)-13-hydroperoxy-9,11-octadecadi enoic acid; 13-HPOT, (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9, 11,15-octadecatrienoic acid; 9-HPOD, (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadi enoic acid; 9-HPOT, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10, 12,15-octadecatrienoic acid.

2. Materials and methods

2.1. Materials

[1-^{14}C]Linoleic (2.1 GBq/mmol) and [1-^{14}C]linolenic (1.95 GBq/mmol) acids were purchased from Amersham International (Amersham, UK) and Institute of Applied Chemistry (St. Petersburg, Russia), respectively. Unlabelled linoleic and linolenic acids, lead tetraacetate as well as soybean lipoxygenase were obtained from Fluka Chemie (Buchs, Switzerland). Soybean lipoxygenase type I-B and [$^{2}H_3$]acetonitrile were purchased from Sigma (St. Louis, MO). Potato tuber lipoxygenase preparation was obtained as described previously [7]. Garlic (*Allium sativum* L.) bulbs were purchased from the local market.

2.2. Substrate preparations

[1-^{14}C](9*Z*,11*E*,13*S*)-13-Hydrop eroxy-9,11-octadecadienoic acid (13-HPOD) and [1-^{14}C]-(9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT) were prepared by incubations of linoleic and linolenic acids, respectively, with soybean lipoxygenase as described previously [8,9]. [1-^{14}C]9-HPOD and [1-^{14}C]9-HPOT were obtained by incubations of linoleate and linolenate, respectively, with potato tuber lipoxygenase as described in [7,9].

2.3. Preparation of garlic bulb juice and incubations with [^{14}C]fatty acids and hydroperoxides

After removing the scale, garlic bulbs (15 g) were homogenized at 0–4°C. The pulp obtained was filtered through 2 layers of Miracloth. The resulting juice (pH ~6.5) was incubated in analytical experiments with 37 kBq of [1-^{14}C]linoleate, [1-^{14}C]linolenate [1-^{14}C]13-HPOD, [1-^{14}C]13-HPOT or [1-^{14}C]9-HPOD (all with specific activities 5.2 MBq/mmol) for 40 min at 25°C. For micro-preparative isolation of compounds III and IV, the juice obtained from 30 g of bulbs was incubated with 56 kBq of [1-^{14}C]13-HPOD or [1-^{14}C]13-HPOT (both with specific activities 520 kBq/mmol).

2.4. Extraction and analysis of products

After each incubation, the reaction mixture was acidified with glacial acetic acid to pH 4–5 and extracted by ethyl acetate/hexane 1:1 and then twice with ethyl acetate. The extracted products were methylated with ethereal diazomethane. Radio-HPLC analyses were performed in main details as described previously [7–9]. The obtained methyl esters were separated by the reversed-phase radio-HPLC on the Octadecyl-Daltosil 100 (5 μm, 4.6 × 250 mm; Serva Feinbiochemica, Heidelberg, Germany) column using linear gradient from 60:40 to 96:4 (v/v) in the solvent mixture methanol/water for 55 min, then 25 min more in isocratic conditions, flow rate 0.4 ml/min. Radioactivity in the eluate was detected by HPLC radiomonitor model 171 (Beckman Instruments, Fullerton, CA) with solid scintillator cell (125 μl). Fractions collected during the reversed-phase HPLC were finally purified on the column LiChrosorb NH_2 (5 μm, 250 × 4.0 mm; E. Merck, Darmstadt, Germany), using the solvent mixture hexane/ethyl acetate 99.5:0.5 (v/v), flow rate 0.4 ml/min.

2.5. Hydrolysis of divinyl ethers

For the examination of ether bond position, the purified samples of products III and IV were subjected to acidic hydrolysis. Solutions of compounds III and IV (0.5 mg each) in 0.75 ml of acetone were mixed with 0.25 ml volume of 20% aqueous HCl. The reaction mixture was stirred at 25°C for 30 min. Then solvents were evaporated under the stream of nitrogen. The residue was dissolved and analysed by the reversed-phase HPLC as described above.

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2.6. Spectral studies

UV topograms were recorded on line during HPLC analyses using RSD 2140 diode array detector (LKB, Bromma, Sweden). Chemical ionization (reagent gas-isobutane) mass spectra of purified metabolites were recorded with Finnigan MAT 212 instrument. The electron impact (70 eV) mass spectra were recorded with high-resolution MKh-1310 spectrometer. The precise mass values were estimated under the computer control, using low boiling perfluorokerosene as a reference standard. All mass spectral records were done using direct sample insertion. [^{1}H]NMR spectra (250 MHz, C^2H_3CN), were recorded with Bruker WM-250 spectrometer. The homonuclear double-resonance experiments were performed with the same instrument.

3. Results and discussion

Incubations of bulb juice with [1-^{14}C]linoleic acid or [1-^{14}C]13-HPOD led to the formation of one predominant product III (isolated as methyl ester, λ_{max} at 248 nm, retention time at ~70 min; see Fig. 2A). The quasi-molecular ion dominated in chemical ionization mass spectrum of this compound: *m/z* (relative intensity, %) 309.244 ($C_{19}H_{33}O_3$) $[M+H]^+$ (100). The following most prominent ions were detected in the electron impact mass spectrum of compound III: *m/z* [ion attribution; relative intensity, %] 308.235 [M^+, $C_{19}H_{32}O_3$; 73]; 177.130 [M^+-$CH_3(CH_2)_3CH$ = CHO-CH_3OH; 26]; 99.081 [M^+-CH = CHCH = $CH(CH_2)_7COOCH_3$; 11]; 81.070 [99.081-H_2O; 100]. [^{1}H]NMR spectrum of compound III is shown in Table 1. The interpretation of spectrum was proved by the data of homonuclear double resonance. The [^{1}H]NMR data are very similar to those for colneleic acid [10,11]. At the same time, the patterns of fragmentation in the electron impact mass spectrum (listed above) suggest that compound III has the ether oxygen between C-12 and C-13. The acidic hydrolysis of compound III led to the single-labelled product, identified by its chemical ionization mass spectrum ($[M+H]^+$ at *m/z* 227.165; $C_{13}H_{23}O_3$), UV spectrum (λ_{max} at 225 nm during RP-HPLC analysis) and RP-HPLC retention data as 10(*E*)-12-oxo-10-dodecenoic acid methyl ester. Thus, the obtained data allow to ascribe the structure of (9*Z*,11*E*,1′*E*)-12-(1′-hexenyloxy)-9,11-dodecadienoic acid methyl ester to compound III (Fig. 1).

The similar non-polar product IV (λ_{max} at 264 nm) was detected upon the incubations of [1-^{14}C]α-linolenic acid or [1-^{14}C]13-HPOT with bulb juice (Fig. 2B). Two most prominent ions were detected in chemical ionization mass spectrum of compound IV methyl ester: *m/z* (element composition; relative intensity, %) 307.228 ($C_{19}H_{31}O_3$) $[M+H]^+$ (100) and 275 [M+H-CH_3OH]$^+$ (27). Peaks of the following most prominent and

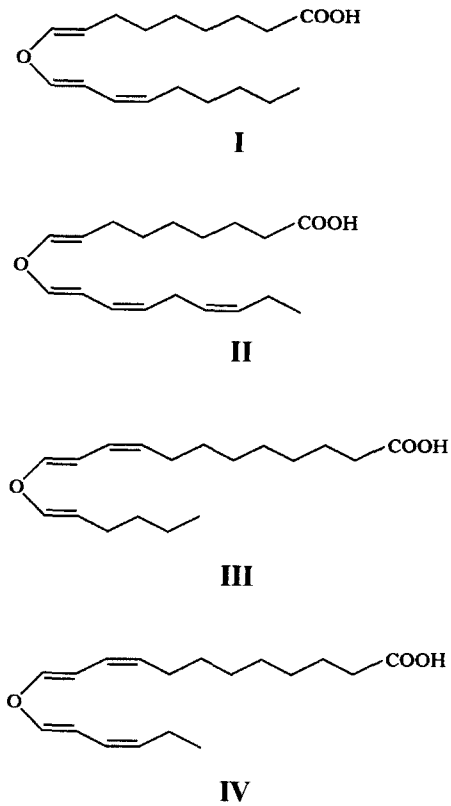

Fig. 1. The structural formulae of compounds I (colneleic acid), II (colnelenic acid), III (etheroleic acid) and IV (etherolenic acid).

characteristic ions are present in the electron impact mass spectrum of metabolite IV: *m/z* [ion attribution; relative intensity, %] 306.220 [M^+, $C_{19}H_{30}O_3$; 100], 207.138 [M^+-CH_3CH_2CH = CHCH = CH-H_2O; 2]; 193.123 [M^+-CH_3CH_2CH = CHCH = $CHCH_3OH$; 2]; 97.065 [M^+-CH = CHCH = $CH(CH_2)_7COOCH_3$; 12]; 81.073 [M^+-OCH = CHCH = $CH(CH_2)_7COOCH_3$; 59]. The [^{1}H]NMR spectra of compounds IV (Table 2) and III (Table 1) in many details are similar. At the same time, the data in Table 2 clearly show that product IV has the double bond at (n-3) position. Thus, metabolite IV is the derivative of linolenic acid. Assignments of all signals of double-bond protons were confirmed by spin-decoupling experiments. The double-bond methins of compound IV due to their symmetric structure were represented in spectrum by four pairs of superposing

Table 1
[^{1}H]NMR spectrum of compound III (250 MHz, C^2H_3CN)

Proton (number of protons)	Chemical shift (δ, ppm)	Multiplicity	Coupling constants (Hz)
H-6′ (3)	0.92	t	6.9 (H-5′)
H-4/7; H-4′; H-5′ (12)	1.33	m	
H-3 (2)	1.59	m	
H-3′ (2)	1.97	m	
H-8 (2)	2.12	m	
H-2 (2)	2.30	t	7.4 (H-3)
H(OMe) (3)	3.63	s	
H-2′ (1)	5.17	dt	12.2 (H-1′); 7.4 (H-3′)
H-9 (1)	5.30	dt	10.3 (H-10); 7.5 (H-8)
H-10 (1)	5.90	ddt	10.3 (H-9); 11.5 (H-11); 1.2 (H-8)
H-11 (1)	6.01	dd	11.5 (H-10); 11.5 (H-12)
H-1′ (1)	6.39	dt	12.2 (H-2′); 1.2 (H-3′)
H-12 (1)	6.65	d	11.5 (H-11)

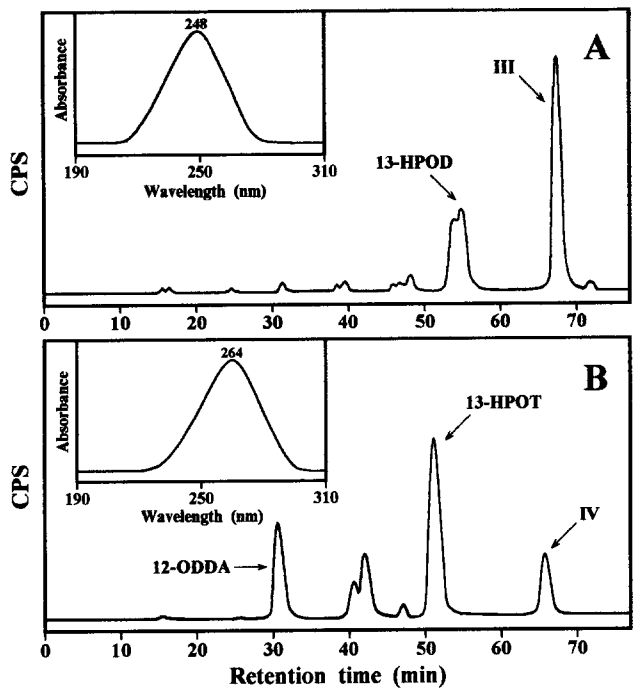

Fig. 2. Reversed-phase radio-HPLC analysis of methyl esters of products of [1-^{14}C]13-HPOD (A) and [1-^{14}C]13-HPOT (B) incubations with juice of garlic bulbs. Peaks III and IV correspond to products III and IV, respectively. 12-ODDA, peak of 9(*Z*)-12-oxo-9-dodecenoic acid. Inserts in A and B: the UV spectra of compounds III and IV, respectively.

signals: H-9/H-4′; H-10/H-3′; H-11/H-2′ and H-12/H-1′. The values of coupling constants are very close to those of colneleic acid [10,11] and compound III. As well as the product III, compound IV upon its acidic hydrolysis is converted into a single-labelled product, identified as 10(*E*)-12-oxo-10-dodecenoic acid methyl ester. Thus, the data obtained allow to ascribe the structure of (9*Z*,11*E*,1′*E*,3′*Z*)-12-(1′,3′-hexadienyloxy)-9,11-dodecadienoic acid to compound IV (Fig. 1).

The names of identified novel divinyl ethers III and IV, dictating by IUPAC nomenclature, – (9*Z*, 11*E*,1′*E*)-12- (1′-hexenyloxy)-9,11-dodeca dienoic and (9*Z*,11*E*,1′*E*,3′ *Z*)-12-(1′,3′-hexadi-enyloxy)-9,11-dodecadienoic acids, respectively – are long and hardly apprehending. Therefore, it seems reasonable to introduce the trivial names. We suggest the trivial names 'etheroleic' and 'etherolenic' acids for compounds III and IV, respectively.

As mentioned in Section 1, colneleic (I) and colnelenic (II) acids were discovered by Galliard and his collaborators in in vitro experiments with potato tubers [3–6]. Up to date compounds Iand II presented the only known examples of natural divinyl ether fatty acids. The enzyme of potato, synthesizing colneleic and colnelenic acids, specifically utilizes 9-HPOD and 9-HPOT as substrates [6]. Garlic, belonging to monocotyledonous species, is philogenetically distant from potato (Dicotyledonae). Thus, the results of present work show that the biosynthesis of divinyl ether fatty acids is not the unique property of potato as previously [1,11,12] assumed. Our results show that the divinyl ether synthase of garlic bulbs, unlike potato enzyme, uses preferentially 13-HPOD and 13-HPOT (the results of incubations with 9-hydroperoxides are not illustrated). The most efficient substrate of garlic enzyme is 13-HPOD (Fig. 2). Such substrate specificity of divinyl ether synthase from garlic bulbs correlates with the regiospecificity of garlic lipoxygenase, producing mostly 13-hydroperoxides (data not shown). The conversion of 13-hydroperoxides into the divinyl ethers III and IV by garlic divinyl ether synthase is probably accomplished by the mechanism of heterolytic rearrangement as depicted in Fig. 3. Such mechanism was proposed previously [1,11] for potato enzyme, synthesizing colneleic (I) and colnelenic (II) acids.

In our experiments the label from [^{14}C]13-hydroperoxides except etheroleic (III) and etherolenic (IV) acids, was incorporated into (9*Z*)-12-oxo-9-dodecenoic acid. Kinetic data (not shown) suggest that divinyl ethers III and IV, being the primary products, are precursors of aldehydes. The rapid rate of etherolenic acid (IV) hydrolysis even hindered its preparation. Synthesis and hydrolysis of compounds III and IV resemble the action of the 'heterolytic' hydroperoxide lyase. This enzyme, widely occurring in plants, catalyzes the chain cleavage in hydroperoxide molecule [1]. In this way 13-HPOD and 13-HPOT are cleaved into hexanal and 3(*Z*)-hexenal (methyl end fragments), respectively, and 12-oxo-9(*Z*)-dodecenoic acid (carboxyl end fragment) in plant leaves [1]. The proposed mechanism of hydroperoxide lyase action [1,11] includes the formation of an intermediary carbocation [1] or oxonium [11] ion with an ether bond. However, any of possible intermediates

Table 2
[^{1}H]NMR spectrum of compound II (250 MHz, CD_3 CN)

Proton (number of protons)	Chemical shift (δ, ppm)	Multiplicity	Coupling constants (Hz)
H-6′ (3)	0.99	dt	7.8 (H-5′); 3.0 (H-4′)
H-4/7; (8)	1.32	m	
H-3 (2)	1.58	m	
H-8, H-5′ (4)	2.15	m	7.5 (H-4′); 7.5 (H-6′)
H-2 (2)	2.30	t	7.4 (H-3)
H(OMe) (3)	3.62	s	
H-9, (1)	5.34	m	10.4 (H-10)*; 7.5 (H-8)*
H-4′ (1)	5.34	m	10.4 (H-3′)*; 7.5 (H-5′)*
H-10 (1),	5.90	m	10.4 (H-9)*; 11.7 (H-11)*
H-3′ (1)	5.90	m	10.4 (H-4′)*; 11.7 (H-2′)*
H-11 (1)	6.09	dd	11.7 (H-10); 11.7 (H-12)
H-2′ (1)	6.09	dd	11.7 (H-1′); 11.7 (H-3′)
H-12 or H-1′ (1)	6.72	d	11.7
H-1′ or H-12 (1)	6.74	d	11.7

*The values of coupling constants $J_{9,10}$ and $J_{1',2'}$ were measured in double-NMR experiments after the suppression of signal at 2.15 ppm (H-8 and H-5′).

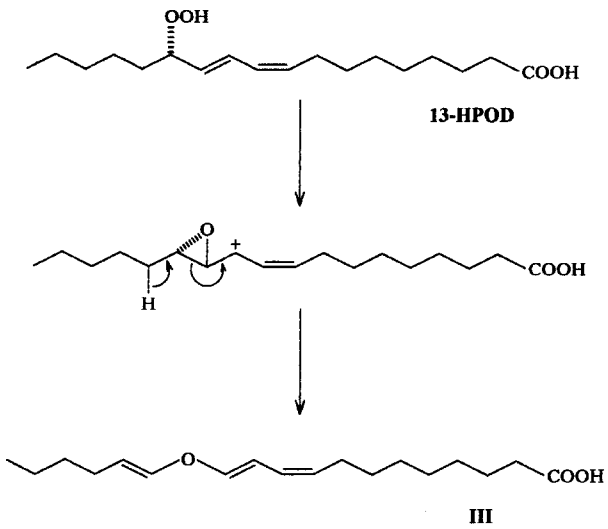

Fig. 3. The proposed mechanism for the conversion of 13-HPOD into the etheroleic acid (III) by garlic enzyme.

have not been isolated previously [1]. Detection of divinyl ether fatty acids, biosynthesized from 13-HPOD and 13-HPOT, allow one to propose that these compounds may be involved as intermediates in 13-hydroperoxide chain cleavage by 'heterolytic' hydroperoxide lyase. Even if this is not the case, reactions, controlled by hydroperoxide lyase and divinyl ether synthase, are evidently neighbouring.

Further work on the characterization of garlic divinyl ether synthase as well as the studies on hydrolysis of etheroleic (III) and etherolenic (IV) acids are in progress in our laboratory.

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