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Divinyl ether synthase from garlic (*Allium sativum* L.) bulbs: sub-cellular localization and substrate regio- and stereospecificity

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Abstract Sub-cellular localization and some properties of 13-hydroperoxide-specific divinyl ether synthase from garlic bulbs were studied. Sub-cellular fractions from garlic bulbs were incubated with [1-^{14}C](9*Z*,11*E*,13*S*)-13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD). The predominant part of divinyl ether synthase activity from garlic bulbs was found in the microsomal fraction. The enzyme utilizes 13(*S*)-HPOD as its preferential substrate. Other hydroperoxides, including 9(*S*)-HPOD, gave much poorer yields of divinyl ethers. Unreacted hydroperoxide after incubation of 13(*R*,*S*)-HPOD with enzyme was composed of up to 94% 13(*R*)-HPOD. Thus, divinyl ether synthase possesses stereoselectivity, utilizing preferentially the (*S*)-enantiomer.

Key words: Divinyl ether synthase localization; Stereospecificity; Regiospecificity

1. Introduction

The lipoxygenase pathway in higher plants is the source of numerous oxylipins (octadecanoids), many of which are of great interest because of their physiological activity [1,2]. There are few known enzymes controlling the secondary conversion of primary lipoxygenase products, fatty acid hydroperoxides. Biosynthesis of the divinyl ethers colneleic (**I**) and colnelenic (**II**) acid by enzyme from potato tubers was considered previously an exotic non-common route for the plant lipoxygenase pathway [3–9]. However, the activity of divinyl ether synthase was detected recently in garlic (*Allium sativum* L.) bulbs [10]. Unlike potato enzyme, utilizing 9-HPOD and 9-HPOT, garlic divinyl ether synthase converts 13-HPOD and (with lesser efficiency) 13-HPOT into the divinyl ethers etheroleic (**III**) and etherolenic (**IV**) acid, respectively.

The present work is concerned with sub-cellular localization and substrate regio- and stereospecificity of divinyl ether synthase from garlic bulbs.

2. Materials and methods

2.1. Substrate preparations

A sample of 13(*S*)-HPOD was prepared by incubation of linoleic acid with type V soybean lipoxygenase (Sigma). Racemic hydroperoxides, 9(*Z*),11(*E*)- and 9(*E*),11(*E*)-13(*R*,*S*)-HPOD, were prepared by autoxidation of 100 mg of linoleic acid for 15 h in oxygen atmosphere. The resulting mixture of racemic hydroperoxides, having *Z*,*E*- and *E*,*E*-double bond geometry, was separated by RP-HPLC. Samples of 9(*Z*),11(*E*)- and 9(*E*),11(*E*)-13(*R*,*S*)-HPOD were collected and used for incubation with divinyl ether synthase.

2.2. Preparation of cell-free fractions from garlic bulbs

Garlic bulbs were homogenized in 0.1 M borate buffer, pH 9.03. The homogenate was filtered through cheesecloth and centrifuged for 16 min at 9300×*g*. The supernatant was decanted and centrifuged for 1 h at 105 000×*g*.

2.3. Incubations, product extraction and HPLC analyses

Substrates were incubated with cell-free fractions from garlic (substrate concentrations and incubation times are specified in each case below). After acidification with 2 N HCl to pH 3.5 products were extracted with diethyl ether. Products were separated as free acids by RP-HPLC on a Macherey-Nagel Nucleosil 5 ODS column (250×4.6 mm), solvent mixture acetonitrile–water–acetic acid (60:40:0.01, v/v), flow rate 1.5 ml/min. Individual divinyl ethers after methylation with diazomethane were separated and purified by normal phase HPLC, two columns Daltosil 100 (250×4.6 mm; Serva Feinbiochemica, Heidelberg), connected in series, elution with a mixture of hexane–ethyl acetate (98.5:1.5, v/v), flow rate 0.8 ml/min.

2.4. Derivatization and analysis of hydroperoxide chirality

Analyses of hydroperoxide chirality were performed in general as described previously [11]. Hydroperoxide remainders after 13(*R*,*S*)-HPOD incubation with enzyme preparations were reduced with sodium borohydride. The resulting hydroxy acid was methylated with diazomethane and propionylated with 0.1 ml propionic anhydride in 0.1 ml of dry pyridine for 15 h at 23°C. Ozonolysis of the propionyl derivative, followed by peracetic acid treatment, afforded 2-propionyloxyheptanoic acid. The diastereomeric amide of this acid, *N*-(2-propionyloxyheptanoyl)-L-phenylalanine methyl ester, was prepared as described in [11]. Chiral and racemic standards of the same amide derivative were prepared from 13(*S*)-HPOD and 13(*R*,*S*)-HPOD, respectively. Analyses of diastereomeric derivatives were performed by GLC on a DB-210 capillary column at 190°C, helium flow rate 36 cm/s.

2.5. Mass spectrometry

Mass spectra were recorded with Hewlett-Packard model 5970B gas chromatography–mass spectrometry system.

3. Results and discussion

As seen in Table 1, the predominant part of divinyl ether synthase activity in garlic bulbs is localized in microsomes. The remaining part of activity was found in the 105 000×*g* supernatant. However, the specific activity of the enzyme in microsomes is much larger than in the 105 000×*g* supernatant (Table 1). These observations are generally consistent with the earlier finding that divinyl ether synthase from potato tubers is a microsomal membrane bound enzyme [6]. In further experiments garlic bulb microsomes were used as an enzyme preparation for incubations.

Incubations of 13(*S*)-HPOD with garlic enzyme afforded a single predominant product (isolated as its methyl ester), iden-

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Abbreviations: 13-HPOD, 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 9-HPOD, 9-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 13-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; HPLC, high performance liquid chromatography; GLC, gas-liquid chromatography; GC-MS, gas chromatography-mass spectrometry; EIMS, electron impact mass spectrum

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Table 1
Distribution of divinyl ether synthase activity in sub-cellular fractions from garlic bulbs

Sub-cellular fraction	13(*S*)-HPOD conversion into acid **III** (%)	Specific activity (μmol/min per mg protein)	Distribution of total activity (%)
Microsomes	70.4	1.47	76
105 000 × *g* supernatant	21.4	0.05	24

0.3 mg of 13(*S*)-HPOD was incubated with sub-cellular fractions, obtained from 1.25 g of garlic bulbs.

tified recently [10] as etheroleic acid (**III**) (9*Z*,11*E*,1′*E*)-12-(1′-hexenyloxy)-9,11-dodecadienoic acid: λ_{max} at 251 nm (EtOH); EIMS, *m/z* [ion attribution, relative intensity, %]: 308 [M^+, 100]; 277 [M^+, CH_3O, 9]; 251 [M^+, C_4H_9, 9], 209 [M^+, $C_6H_{11}O$, 3], 177 [M^+, $C_6H_{11}O$, CH_3OH, 28], 165 [M^+, $(CH_2)_6COOCH_3$, 42]; 159 [177, H_2O, 31]. Separation of 13(*S*)-HPOD products by reversed phase HPLC revealed one more minor peak along with etheroleic acid (**III**) itself (approximate ratio to etheroleic acid 1:10), exhibiting similar ultraviolet absorbance, λ_{max} at 250 nm (EtOH). Upon methylation with diazomethane this compound was analyzed by GC-MS. This analysis revealed two nearly equal peaks. Their mass spectra were identical to that of etheroleic acid. These products were identified by their ^{1}H-NMR spectral data as 9(*E*),11(*E*),1′(*E*)- and 9(*Z*),11(*Z*),1′(*E*)-isomers of compound **III** (results not illustrated).

As seen from Table 2, 13(*S*)-HPOD is the best substrate for garlic divinyl ether synthase. With lesser efficiency the enzyme converts 13(*S*)-HPOT into etherolenic acid (**IV**) [10]. Unline divinyl ether synthase from potato tubers, the garlic enzyme is ineffective with 9(*S*)-HPOD (Table 2). Thus, the position of the hydroperoxy group has critical importance. The observed substrate specificity of garlic divinyl ether synthase correlates with lipoxygenase regiospecificity. As we have recently found (Grechkin et al., to be published), garlic lipoxygenase possesses strong regio- and stereospecificity, transforming linoleate and linolenate into 13(*S*)-hydroperoxides.

Upon incubation of [$^{18}O_2$-hydroperoxy]13(*S*)-HPOD one of the two ^{18}O atoms was entirely incorporated into the ether bond of etheroleic acid (**III**) (result not illustrated). These data show that epoxyallylic cation, resulting from protonation-dehydration of original hydroperoxide (Fig. 1), is involved as an intermediate in divinyl ether formation.

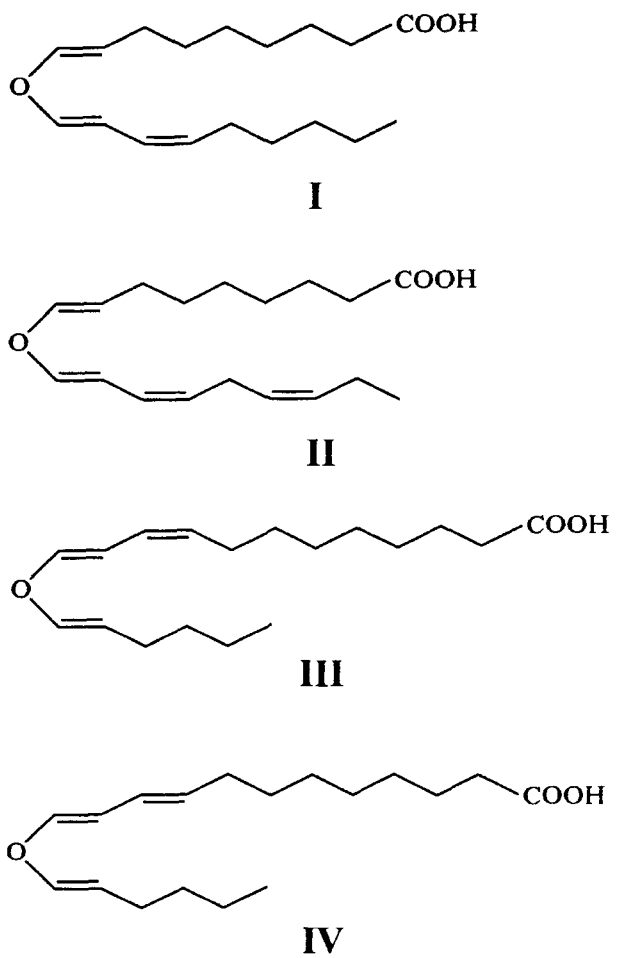

Fig. 1. The structural formulae of divinyl ethers: colneleic (**I**), colnelenic (**II**), etheroleic (**III**) and etherolenic (**IV**) acids.

Table 2
Conversion of different hydroperoxides into divinyl ethers by garlic enzyme

Substrate	Product	Conversion, μmol per mg protein[a]
13(*S*)-HPOD	Etheroleic acid (**III**)	7.69
13(*R,S*)-HPOD	Etheroleic acid (**III**)	2.91
13(*S*)-HPOT	Etherolenic acid (**IV**)	1.42
9(*S*)-HPOD	Colneleic acid (**I**)	not detected

[a]Each substrate (0.5 mg) was incubated with microsomes, equivalent to 0.25 mg protein. Quantification was performed by measurement of peak areas in RP-HPLC chromatograms recorded with ultraviolet detection at 210 nm.

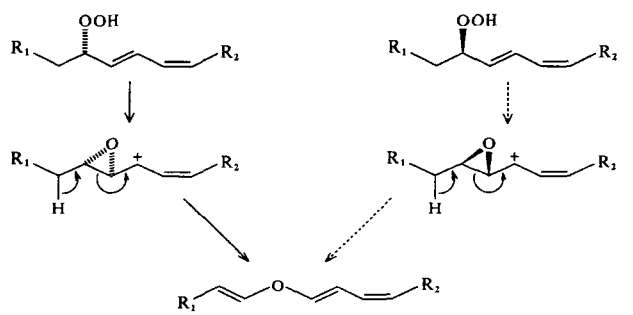

Fig. 2. Stereospecificity of 13(*R,S*)-HPOD utilization by garlic divinyl ether synthase. R_1=*n*-butyl; R_2=-$CH_2(CH_2)_6COOH$.

Table 3
Stereospecificity of 13(*R,S*)-HPOD utilization by garlic divinyl ether synthase

Substrate	Conversion of substrate into divinyl ether **III**(%)	Stereocomposition of unreacted hydroperoxide	
		13(*R*)	13(*S*)
13(*R,S*)-HPOD (*Z,E*)[a]	31.6	70.1	29.9
13(*R,S*)-HPOD (*E,E*)[a]	10.4	58.9	41.1
13(*R,S*)-HPOD (*Z,E*)[b]	50.4	87.8	22.2
13(*R,S*)-HPOD (*Z,E*)[c]	74.6	94.4	5.6

All substrates were incubated with microsomal suspensions for 30 min at 22°C.
[a]13(*R,S*)-HPOD (*Z,E*) or 13(*R,S*)-HPOD (*E,E*) (1 mg each) was incubated with a suspension of microsomes prepared from 3 g of garlic bulbs.
[b]13(*R,S*)-HPOD (*Z,E*) (0.5 mg) was incubated with a suspension of microsomes prepared from 3 g of garlic bulbs.
[c]13(*R,S*)-HPOD (*Z,E*) (0.5 mg) was incubated with a suspension of microsomes prepared from 3 g of garlic bulbs.

Racemic hydroperoxide 13(*R,S*)-HPOD was converted into etheroleic acid (**III**) more slowly than chiral 13(*S*)-HPOD. The remainder of the unreacted 13(*R,S*)-HPOD after incubation with enzyme was subjected to stereospecific analysis (Table 3). This showed that the remaining hydroperoxide was composed of up to 94% 13(*R*)-HPOD enantiomer. This result shows that 13(*R*)-HPOD is utilized much more slowly than 13(*S*)-HPOD. At the same time, 13(*R,S*)-HPOD is utilized almost completely when it is incubated with an excess amount of enzyme. Thus, the enzyme utilizes 13(*S*)-HPOD preferentially, but this requirement to substrate chirality is not absolute (Fig. 2). As found previously, 'heterolytic' hydroperoxide lyase from tea leaves possesses a similar preference to 13(*S*)-HPOT when incubated with 13(*R,S*)-HPOT [1]. Unlike these two enzymes, flax seed allene oxide synthase specifically utilizes only 13(*S*)-HPOT, being not able to transform 13(*R*)-HPOT [1].

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