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Specificity of Oxidation of Linoleic Acid Homologs by Plant Lipoxygenases

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Abstract—The lipoxygenase-catalyzed oxidation of linoleic acid homologs was studied. While the linoleic acid oxidation by maize 9-lipoxygenase (9-LO) specifically produced (9*S*)-hydroperoxide, the dioxygenation of (11*Z*,14*Z*)-eicosadienoic (20:2) and (13*Z*,16*Z*)-docosadienoic (22:2) acids by the same enzyme lacked regio- and stereospecificity. The oxidation of 20:2 and 22:2 by 9-LO afforded low yields of racemic 11-, 12-, 14-, and 15-hydroperoxides or 13- and 17-hydroperoxides, respectively. Soybean 13-lipoxygenase-1 (13-LO) specifically oxidized 20:2, 22:2, and linoleate into (ω6*S*)-hydroperoxides. Dioxygenation of (9*Z*,12*Z*)-hexadecadienoic acid (16:2) by both 9-LO and 13-LO occurred specifically, affording (9*S*)- and (13*S*)-hydroperoxides, respectively. The data are consistent with the "pocket theory of lipoxygenase catalysis" (i.e. with the penetration of a substrate into the active center with the methyl end first). Our findings also demonstrate that the distance between carboxyl group and double bonds substantially determines the positioning of substrates within the active site.

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The lipoxygenase pathway of plants is a source of various oxylipins, which play roles of signaling mediators, growth regulators, and endogenous protectors from both biogenic and abiogenic stress agents [1]. The key enzymes of this pathway are lipoxygenases (EC 1.13.11.12). These monomeric proteins with molecular masses of 94-104 kDa contain one non-heme iron atom per protein molecule and catalyze dioxygenation of polyenoic fatty acids to form corresponding hydroperoxy derivatives. Almost all plant lipoxygenases are highly regio- and stereospecific in catalysis of linoleic and α-linolenic acids and convert them into either (9*S*)-hydroperoxides ((*n* – 2)-type lipoxygenases) or (13*S*)-hydroperoxides ((*n* + 2)-type lipoxygenases) [2].

The problem of control over lipoxygenase regio- and stereospecificity is not yet resolved. Three mutually exclusive hypotheses on interaction of lipoxygenases with substrate exist: "inverse", "pocket", and "carboxylate" ones [3-6]. According to the first hypothesis, a fatty acid molecule can enter the lipoxygenase active center with either its methyl or carboxyl end. Regiospecificity of the enzyme activity is therewith determined by substrate inversion [3, 4]. The "pocket" hypothesis implies the substrate entry to the lipoxygenase active center with the methyl end only, whereas the "carboxylate" one – with carboxyl end only. In these cases, regiospecificity of lipoxygenase catalysis does not depend on substrate orientation in the enzyme active center, but is determined by the primary sequence and conformation of the lipoxygenase molecule [5, 6].

Various approaches and methods have been used to solve the problem of control over lipoxygenase regio- and stereospecificity: analysis of pH effect on regiospecificity of lipoxygenase catalysis [4], X-ray structure analysis [7], site-directed mutagenesis [5, 6, 8-12], and oxidation of complex lipids [13-15] and ω-modified fatty acids [16, 17]. Nonetheless, the available data do not unambiguously substantiate one of the abovementioned hypotheses.

Abbreviations: GC-MS, gas chromatography–mass spectrometry; HPLC, high performance liquid chromatography; 9-LO, maize 9-lipoxygenase CSSAP 92; 13-LO, soybean lipoxygenase-1.

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The present work was undertaken to ascertain how regio- and stereospecificity of lipoxygenase-catalyzed oxidation depend on the chain length of dienoic fatty acids and double bond positions.

MATERIALS AND METHODS

Chemicals and enzymes. Linoleic acid, 20:2, and 22:2, as well as soybean lipoxygenase-1 (type V) (13-LO) were purchased from Sigma (USA), and 16:2 was purchased from Larodan (Sweden). Sodium borohydride, platinum catalyst, and reagents for silylation were purchased from Fluka (Switzerland).

Recombinant maize 9-lipoxygenase CSSAP 92 (9-LO) was prepared as described earlier [18]. The recombinant protein was purified at 4°C, as follows. 9-LO was precipitated with $(NH_4)_2SO_4$ (20-50% saturation) and centrifuged at 18,000*g* for 20 min. The pellet was dissolved in buffer A containing 0.01 M Tris-HCl, pH 8.0, and 0.01% Triton X-100 and dialyzed overnight against the same buffer. The dialyzed sample was applied onto a column (11 × 1.5 cm) with Q-Sepharose and eluted with linear gradient of NaCl (0-0.35 M; elution rate 1 ml/min). Fractions containing lipoxygenase were dialyzed overnight against buffer A containing 20% $(NH_4)_2SO_4$ and applied onto a column (16.5 × 1.0 cm) with Octyl-Sepharose. The protein was eluted with a linear gradient of $(NH_4)_2SO_4$ (20-0%; elution rate 1 ml/min). Protein was determined by the method of Bradford as described in [14]. The purified 9-LO was frozen in liquid nitrogen and stored at –85°C.

Kinetic experiments. A standard analytical mixture (2 ml) containing 0.3 mM of either 16:2, 18:2, 20:2, or 22:2 in 50 mM sodium phosphate buffer (pH 9.0 or 7.0) was preliminarily saturated with oxygen for 3 min. Reaction was initiated by addition of 5 μkat of either 9-LO or 13-LO and continued for 40 sec at 20°C. Enzymatic activity was determined from absorbance at 234 nm on a Cary 50 Bio spectrophotometer (Varian, Australia). The linear region of kinetic curve corresponding to the stationary reaction phase was used for calculation of enzymatic reaction rate. Six independent experiments were performed for each specified variant. The mean values and standard deviations are given.

Incubation of fatty acids with lipoxygenases. Either 16:2, 18:2, 20:2, or 22:2 (0.5 mg) was incubated with 25 μkat of either 9-LO or 13-LO in 10 ml of 0.05 M Tris-HCl buffer (pH 9.0) at 4°C for 30 min with continuous oxygen bubbling. The reaction was terminated by addition of glacial acetic acid until the pH of the mixture reached 4.5. Reaction products were thrice extracted with a mixture of equal volumes of ethyl acetate and hexane.

Derivatization of products. Following vacuum evaporation of solvents, the products were reduced with sodium borohydride and methylated with diazomethane. Methyl ethers of the products were reduced over PtO_2 and trimethylsilylated by treatment with silanizing mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane (2 : 1 : 2 v/v).

HPLC. Methyl ethers of product hydroxy-derivatives were separated by straight-phase HPLC under isocratic conditions with two tandem Separon SIX columns (5 μm; 3.2 × 150 mm; Tessek, Czech Republic), solvent system of hexane–propane-2-ol (98.5 : 1.5 v/v), and flow rate of 0.4 ml/min. Fractions of hydroxy acids were collected and analyzed by chiral-phase HPLC under isocratic conditions with a Chiralcel OD-H column (5 μm; 4.6 × 250 mm; Daicel Chemical Industries, France), solvent system of hexane–propane-2-ol (97 : 3 v/v), and flow rate of 0.4 ml/min.

Mass-spectrometry. Methyl/TMS (trimethylsilyl)-derivatives prepared by reduction of hydroperoxides with sodium borohydride, methylation, catalytic hydrogenation of double bonds over PtO_2, and trimethylsilylation were analyzed by gas chromatography–mass spectrometry (GC-MS) as described earlier [19]. The column temperature during analyses was elevated from 120 to 240°C with heating rate of 10°C/min.

RESULTS

Kinetic experiments on oxidation of 20:2 and 22:2 demonstrated that 13-LO can oxidize both substrates to form hydroperoxy derivatives (increase in absorbance at 234 nm was detected) with nearly equal rates (1.85 ± 0.38 and 1.81 ± 0.33 μmol/min, respectively) compared with the rate of 18:2 dioxygenation (1.92 ± 0.23 μmol/min). In contrast, 9-LO at pH 7.0, which is commonly optimal for the activity of this enzyme, did not utilize 22:2 and catalyzed 20:2 dioxygenation 20-fold slower (0.1 ± 0.08 μmol/min) than 18:2 dioxygenation (2.01 ± 0.18 μmol/min). This might be associated with significantly lower polarity and water solubility at neutral pH of higher 18:2 homologs compared with 18:2. To even this negative influence, we evaluated the rates of 20:2 and 22:2 dioxygenation at pH 9.0, that is, under circumstances of higher ionization of these fatty acids and, correspondingly, higher accessibility of them to lipoxygenases. Under these conditions 9-LO dioxygenated 20:2 and 22:2 55- and 10.5-fold slower (4.81 ± 0.25 and 25.2 ± 1.26 nmol/min, respectively) than 18:2 (264 ± 19.4 nmol/min), respectively.

To reveal regiospecificity of 9-LO and 13-LO in oxidation of higher 18:2 homologs, we incubated these lipoxygenases with 20:2 and 22:2 at pH 9.0 and analyzed the reaction products in the form of methyl/TMS-ethers by GC-MS. We found that 13-LO converted 22:2 into products *1* and *2* (Fig. 1a). Oxidation of 22:2 catalyzed by 9-LO resulted in formation of the minor product *3* together with *1* and *2* (Fig. 1b). The substances *1*, *2*, and

3 exhibited characteristic mass spectra and fragmentation patterns (Fig. 1, c-e) allowing identification of these substances as methyl/TMS-ethers of 13-, 17-, and 18-hydroxydodecanoic acid, respectively. Thus, 22:2 is dioxygenated by 13-LO preferentially at C-17 position, which corresponds to (n + 2)-type oxidation characteristic of this enzyme [12]. 9-LO dioxygenates 22:2 preferentially at C-13 and C-17, which is indicative of the loss of the regiospecificity of the enzyme.

Analysis of 22:2 oxidation products in the form of methyl ethers by chiral-phase HPLC showed that 98% of the 17-hydroperoxide produced by 13-LO was (*S*)-enantiomer (table). The 13-hydroperoxide of 22:2 produced by this enzyme as well as all main products of 22:2 dioxygenation by 9-LO were racemic mixtures (table).

Similar data were obtained when 20:2 was oxidized by lipoxygenases 9-LO and 13-LO. The major product of 20:2 conversion catalyzed by 13-LO was substance *7* (Fig.

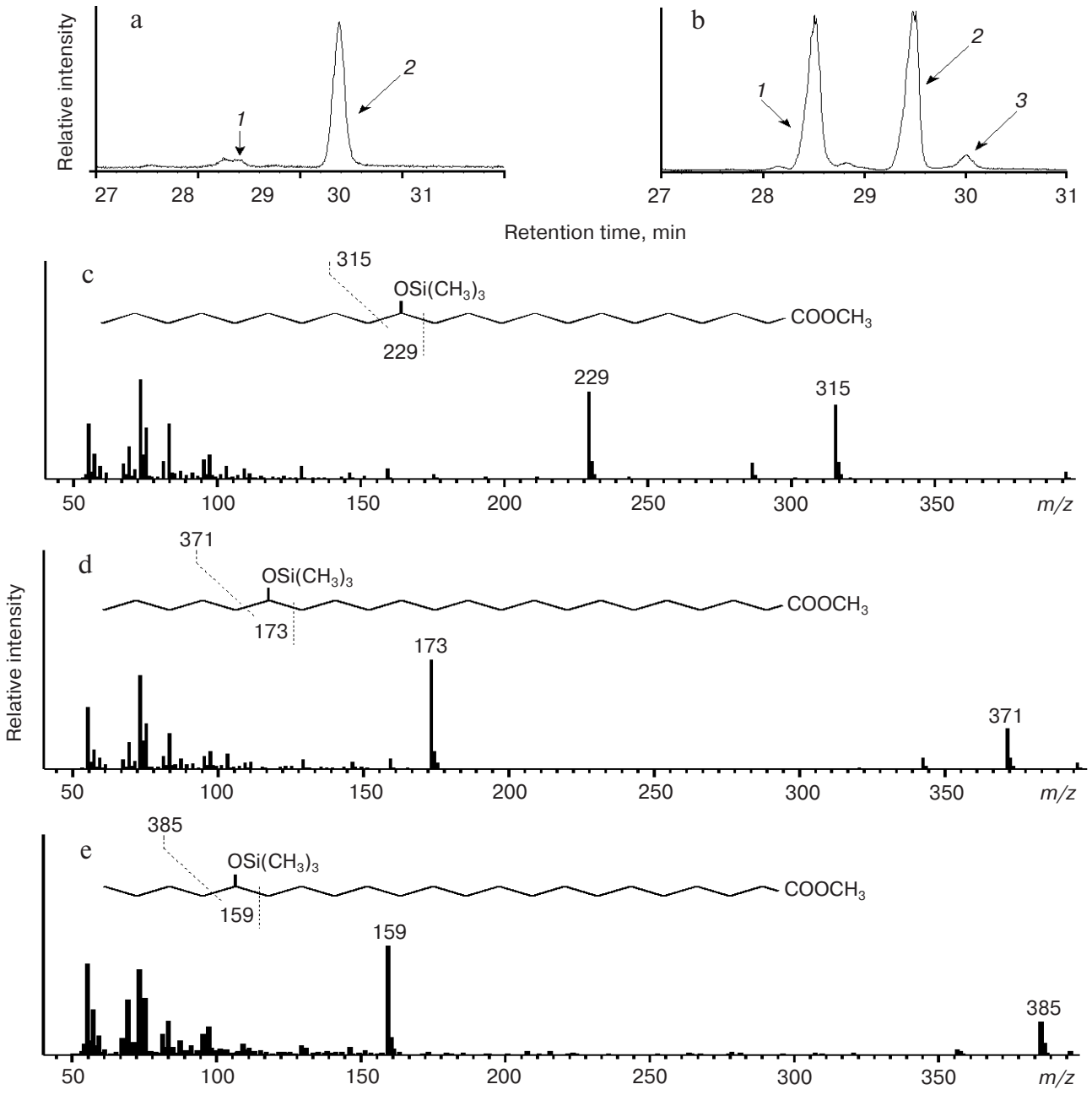

Fig. 1. GC-MS analysis of products of 22:2 oxidation by 13-LO (a) and 9-LO (b). The products were extracted, reduced with $NaBH_4$, methylated, hydrogenated over PtO_2, trimethylsilylated, and analyzed by GC-MS as described in "Materials and Methods". a, b) Total ion chromatograms; c, d, e) mass spectra and fragmentation patterns of substances *1*, *2*, and *3*.

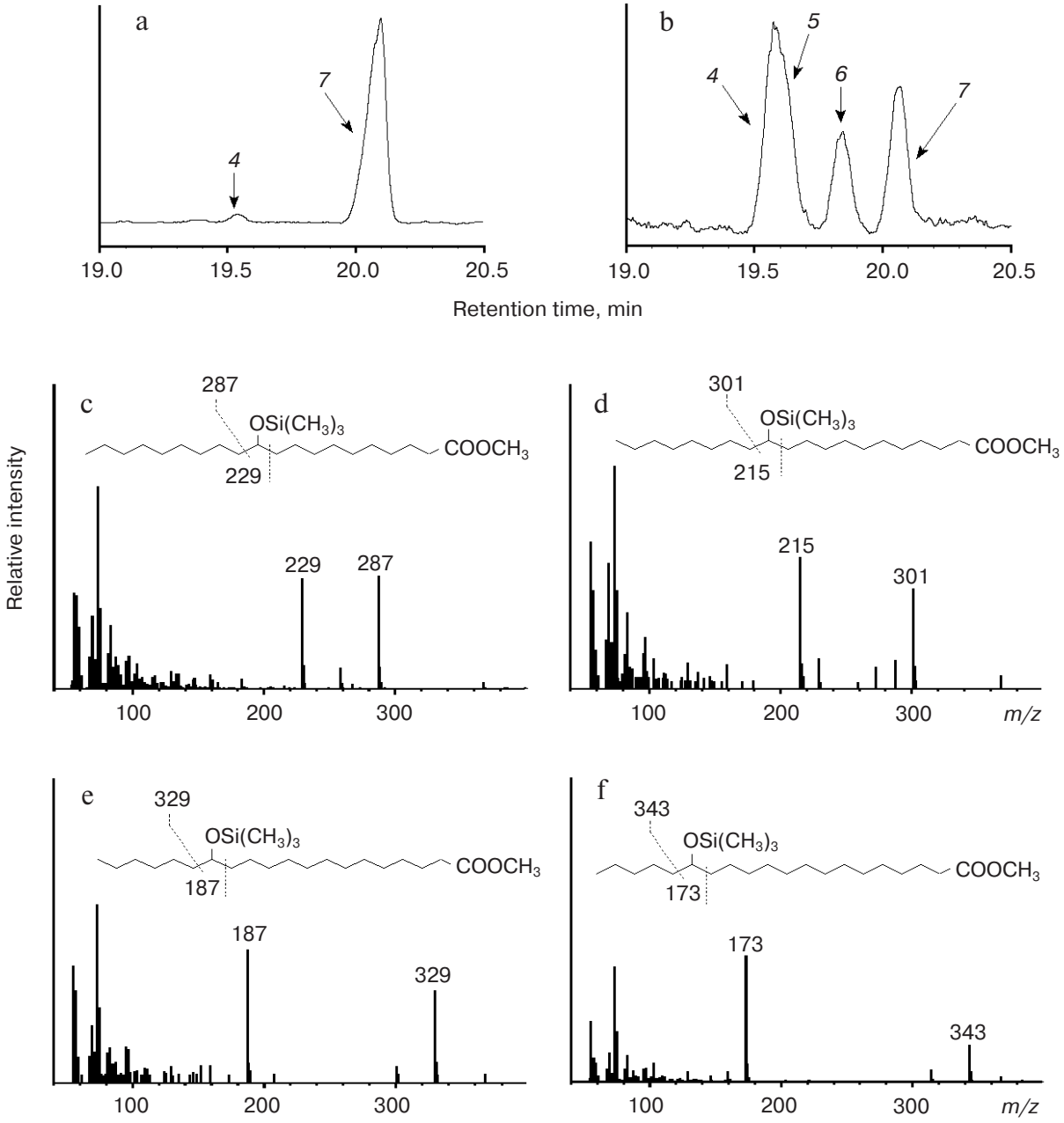

Fig. 2. GC-MS analysis of products of 20:2 oxidation by 13-LO (a) and 9-LO (b). The products were extracted, reduced with $NaBH_4$, methylated, hydrogenated over PtO_2, trimethylsilylated, and analyzed by GC-MS as described in "Materials and Methods". a, b) Total ion chromatograms; c, d, e, f) mass spectra and fragmentation patterns of substances *4*, *5*, *6*, and *7*.

2a). Also, formation of the minor product *4* was observed. Oxidation of 20:2 by 9-LO led to formation of products *4* and *5*, which were not completely separated on the chromatographic column, and products *6* and *7* (Fig. 2b). The ratio between these products was the same at both pH 7.0 (data not shown) and pH 9.0 (Fig. 2b). Mass spectra and fragmentation patterns of substances *4*, *5*, *6*, and *7* (Fig. 2, c-f) allowed their unambiguous identification as methyl/TMS-ethers of 11-, 12-, 14-, and 15-hydroxyeicosanoic acid, respectively. The data demonstrate that 13-LO dioxygenates 20:2 preferentially at C-15 position, which corresponds to the (n + 2)-type oxidation. The oxidation of 20:2 catalyzed by 9-LO was accompanied by the loss of enzyme regiospecificity. Oxidation of 20:2 (Fig. 2b) was far less regiospecific than that of 22:2 (Fig. 1b).

The data of chiral-phase HPLC showed that 13-LO produced 15-hydroperoxide of 20:2 preferentially in (*S*)-configuration, whereas other products formed by lipoxygenases 9-LO and 13-LO from 20:2 were racemic mixtures (table).

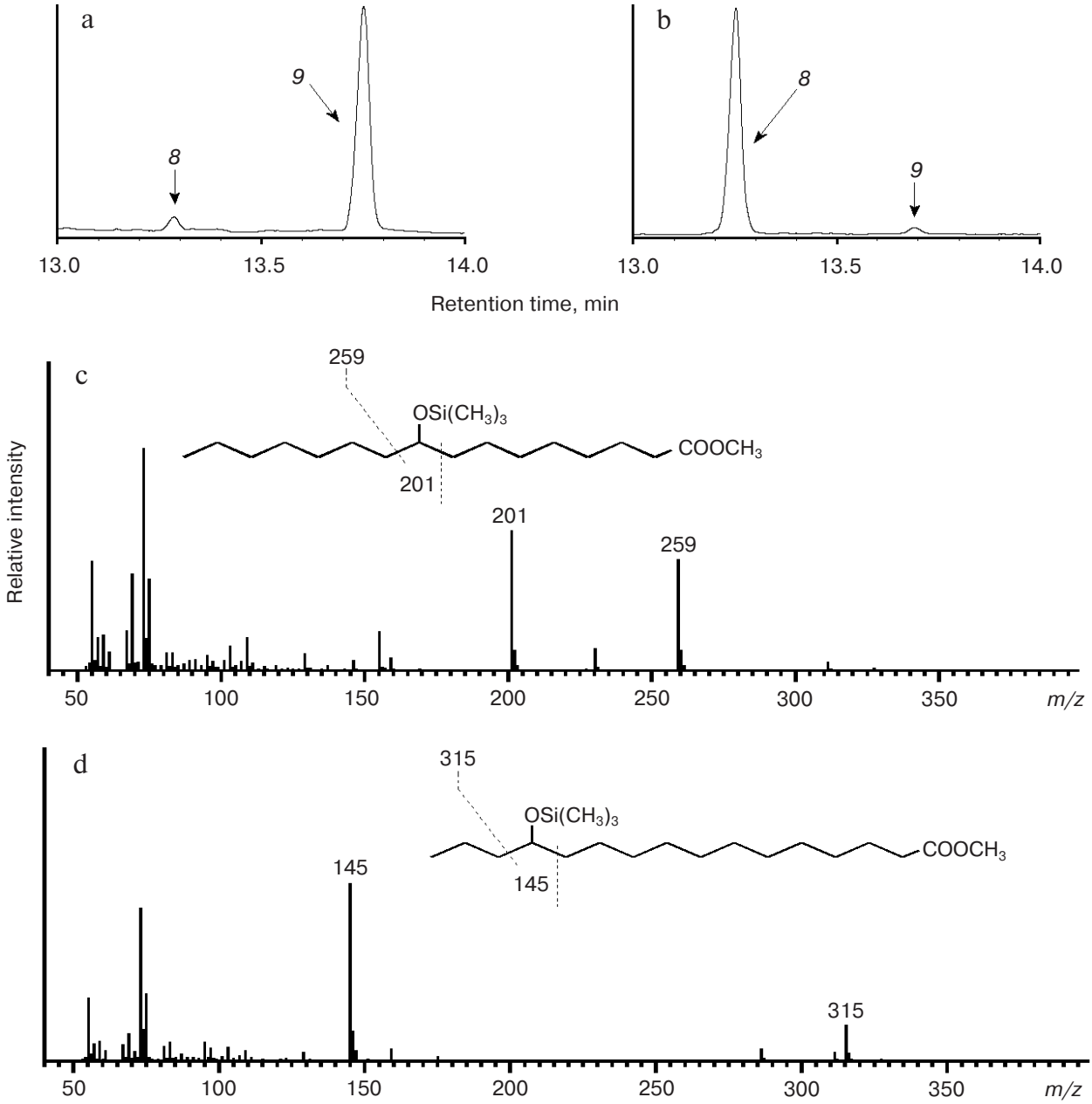

Fig. 3. GC-MS analysis of products of 16:2 oxidation by 13-LO (a) and 9-LO (b). The products were extracted, reduced with $NaBH_4$, methylated, hydrogenated over PtO_2, trimethylsilylated, and analyzed by GC-MS, as described in "Materials and Methods". a, b) Total ion chromatograms; c, d) mass spectra and fragmentation patterns of substances *8* and *9*.

Unlike higher 18:2 homologs, 16:2 was oxidized by 9-LO and 13-LO with equal rates comparable with that of 18:2 oxidation. The degree of 16:2 conversion by lipoxygenases 9-LO and 13-LO into corresponding hydroperoxy derivatives was maximal at pH 7.0 and 9.0, respectively. GC-MS analysis of products of 16:2 incubation with 13-LO at pH 9.0 revealed preferential conversion of this substrate into product *9* (Fig. 3a). Besides, about 5% of substance *8* was produced (Fig. 3a). 9-LO converted 16:2 into the same substances, but in this case the major product was substance *8* (97%; Fig. 3b). The data of mass spectroscopy of substances *8* and *9* allowed their identification as methyl/TMS-ethers of 9- and 13-hydroxyhexadecanoic acid, respectively. Thus, both 9-LO and 13-LO possess the same regiospecificity as they do upon oxidation of 18:2. Using chiral-phases HPLC, we showed that major hydroperoxides produced by both 9-LO and 13-LO from 16:2 have (*S*)-configuration, whereas minor ones are racemic (table). The stereospecificity of 9-LO and 13-LO observed in oxidation of 16:2 is in complete agree-

Stereospecificity of oxidation of linoleic acid and its homologs by lipoxygenases 9-LO and 13-LO

Substrate	Enzyme	Ratio between stereoisomers of oxidation products, %				
		9R/9S	11R/11S	13R/13S	15R/15S	17R/17S
16 : 2	9-LO 13-LO	1 : 99 48 : 52		61 : 39 3 : 97		
18 : 2	9-LO 13-LO	1 : 99 49 : 51		62 : 38 2 : 98		
20 : 2	9-LO 13-LO		50 : 50 50 : 50		50 : 50 3 : 97	
22 : 2	9-LO 13-LO			49 : 51 49 : 51		50 : 50 2 : 98

ment with stereospecificity of these enzymes in oxidation of 18:2 (table).

DISCUSSION

For the most correct interpretation of our data, we have schematically represented all possible variants of interaction of 22:2 with the active center of lipoxygenases (Fig. 4). An attempt to construe the data on lipoxygenase-catalyzed 22:2 oxidation from the point of view of the "inverse" or the "carboxylate" hypothesis (Fig. 4, a and b) led to the conclusion that the prochiral center of this substrate entered insufficiently deeply into the lipoxygenase active center. Moreover, the sites of oxygen attachment (C-17 and C-18) are very distantly localized. These difficulties can be avoided when the results of 22:2 oxidation are interpreted from the "pocket" hypothesis point of view (Fig. 4c). Similar conclusions can be drawn from the analysis of the data on 20:2 oxidation by lipoxygenases 9-LO and 13-LO. The data suggest that both 20:2 and 22:2 enter the active centers of 9- and 13-lipoxygenases with their methyl ends first. Thus, the difference in regiospecific activity between 9-LO and 13-LO results from different organization of their active centers rather than from substrate inversion in the enzyme active centers.

The loss of regiospecificity of 9-LO in oxidation of higher 18:2 homologs is probably associated with the necessity of anchoring of the carboxyl group on the lipoxygenase surface near the entrance to the enzyme active center for precise positioning of the substrate relative to the ferric atom realizing the removal of a hydrogen atom from the prochiral center of the substrate. According to 3D models designed for a number of mammalian lipoxygenases, the positively charged arginine or lysine groups localized at the entrance to the enzyme active center can act as such anchors [7, 9].

The data on 9,12-16:2 oxidation demonstrate that shortening of the distance between the methyl end and double bonds (when the double bonds are kept at unaltered position relative to the carboxyl group) does not alter the position-specificity of the oxidation. Regio- and stereospecificity of 9-LO and 13-LO in the substrate oxidation remain the same as in 18:2 oxidation. Thus, the position specificity of lipoxygenases is determined not only by the extent of submergence of the substrate into the active center, as supposed earlier [9], but largely by the distance between the carboxylate "anchoring" site and the iron atom.

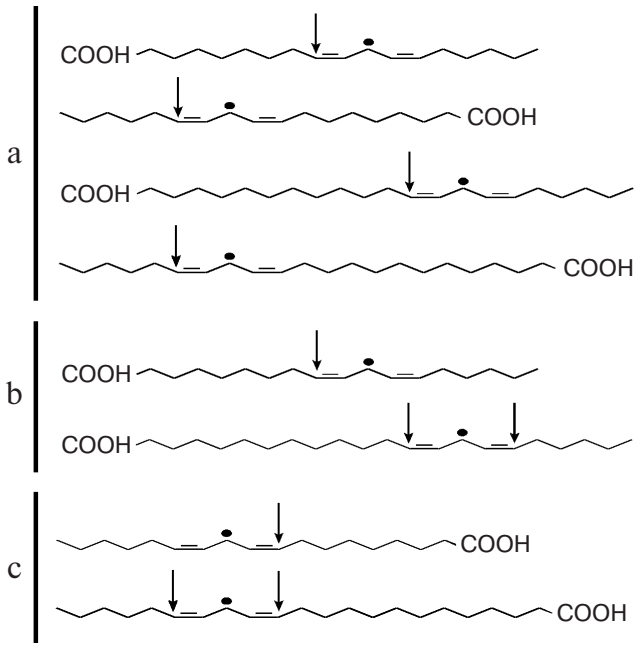

Fig. 4. Oxidation of 18:2 and 22:2 by the recombinant maize 9-lipoxygenase. Interpretation of the data on the basis of "inverse" (a), "carboxylate" (b), and "pocket" (c) hypotheses of lipoxygenase-catalyzed oxidation. The "bottom" of the lipoxygenase active center is designated by the vertical on the left, prochiral centers of lipoxygenases by points, and the experimentally observed positions of dioxygenation by arrows.

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