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In vitro oxygenation of soybean biomembranes by lipoxygenase-2

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Abstract

The ability of soybean lipoxygenases-1 and -2 to oxygenate biomembranes isolated from soybean seedlings has been investigated. Constituents of the lipid bilayer were analyzed by means of reversed phase and chiral phase high performance liquid chromatography, gas chromatography/mass spectrometry, high performance thin layer chromatography and uv spectroscopy. Evidence is presented that soybean lipoxygenase-2, at variance with the type-1 enzyme, oxygenates the esterified unsaturated fatty acid moieties in biomembranes, whereas membrane-embedded free unsaturated fatty acid moieties were not a suitable substrate for either isoenzyme. The oxygenation products derived from the biomembranes were the 9- and 13-hydroperoxides of linoleic acid residues, in a molar ratio of 1.0 to 1.7, and the 9- and 13-hydroperoxides of α-linolenic acid residues, in a molar ratio of 1.0 to 0.1. The *R/S* ratios of 13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid and 9-hydroperoxy-10*E*,12*Z*,15*Z*-octadecatrienoic acid were found to be 0.5 and 25.0, respectively. These stereospecificity values were much higher than those of hydroperoxides isolated after incubation of lipoxygenase-2 with non-membraneous fatty acids or their methyl esters. The hydroperoxy fatty acids produced were distributed in neutral lipids and phospholipids isolated from soybean membranes, the former being oxidized to a larger extent. Furthermore, both intracellular and plasma membranes were substrates for the enzymic oxygenation, with a preference for those of chloroplasts followed by those of Golgi apparatus, endoplasmic reticulum, plasma membrane and mitochondria. These data point towards a different action of the two lipoxygenases in soybean cells. We suggest that the type-2 enzyme plays a role in the in vivo remodelling of biomembranes. The physiological relevance of these findings is discussed.

Key words: Biomembrane; Lipoxygenase-1; Lipoxygenase-2; (*G. max*)

1. Introduction

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) comprise a class of ubiquitous [1–3] non-heme iron-containing dioxygenases, which dioxygenate unsaturated fatty acids with one or more 1*Z*,4*Z*-pentadiene systems to yield *Z*,*E*-conjugated hydroperoxy fatty acids. Soybeans (*Glycine max*) contain several lipoxygenases, the isoenzymes-1 and -2 being the best characterized ones [4]. These enzymes differ in pH optimum, substrate specificity, regio- and stereospecificity and kinetic features [4–6]. Both have been implicated in a variety of processes, like fruit ripening [5], seed germination [7], plant senescence [8], response to plant pathogens [9], wounding [10], anoxia [11], ozone stress [12], thermal injury [12,13] and control of cell viability [14]. The involvement in such a range of different phenomena might point to the existence of a common denominator related to lipoxygenase activity. Interestingly, soybean lipoxygenases have been reported to oxygenate isolated components of biomembranes [15–19]. In this paper, the ability of soybean lipoxygenases-1 and -2 to oxygenate intact soybean biomembranes has been investigated.

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Abbreviations: 9*S*-HOT, 9*S*-hydroxy-10*E*,12*Z*,15*Z*-octadecatrienoic acid; 13*S*-HOT, 13*S*-hydroxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid; 9*S*-HOD, 9*S*-hydroxy-10*E*,12*Z*-octadecadienoic acid; 13*S*-HOD, 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid; RP-HPLC, reversed-phase high-performance chromatography; GC/MS, gas chromatography/mass spectrometry; TMS, trimethylsilyl; HPTLC, high-performance thin-layer chromatography.

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2. Materials and methods

Chemicals were of the purest analytical grade. Authentic 9*S*-HOT, 13*S*-HOT, 9*S*-HOD and 13*S*-HOD were biosynthesized as described [20]. Racemic mixtures of 9-/13-HOT and 9-/13-HOD were produced by autoxidation of α-linolenic and linoleic acids, respectively [21]. Linoleic and α-linolenic acids, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine were purchased from Sigma. Percoll was from Pharmacia.

2.1. Enzymes

Lipoxygenases-1 and -2 were purified from soybeans as reported [22]. Enzyme activity was measured polarographically at 25°C, in a solution of 1.8 mM linoleic acid in air-saturated 0.1 M sodium borate buffer (pH 9.0) (lipoxygenase-1), or 0.1 M sodium phosphate buffer (pH 6.8) (lipoxygenase-2). Protein concentration was determined as described [23], using bovine serum albumin as a standard. Specific activities were 225 U/mg protein, for lipoxygenase-1, and 35 U/mg protein, for lipoxygenase-2. The type-2 enzyme retained its full activity when assayed in the buffer used to resuspend membranes, i.e., 10 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 1 M NaCl [24], while the specific activity of the type-1 enzyme at the pH of this buffer decreased to 67.5 U/mg protein.

2.2. Plant material and membrane isolation

Soybeans (*Glycine max* (L.) Merrill) were grown at 22°C in a greenhouse with a photoperiodic regime of 14 h of daylight and 10 h of darkness. 10-day-old seedlings were homogenized to isolate a whole membrane fraction (referred to as membrane pool) [24]. The endoplasmic reticulum and the plasma membranes were further purified from the membrane pool by sucrose gradient ultracentrifugation [24]. Subcellular fractionation was performed by Percoll or sucrose gradient centrifugation, in order to isolate intact chloroplasts [25], mitochondria [26] and Golgi apparatus [27] from soybeans.

2.3. Incubation of membranes with lipoxygenases

Membranes from the different fractions were suspended in 10 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 1 M NaCl [24], at a final concentration of 3 mg of protein/ml. Each suspension (3 ml) was incubated with either 3.4 U of lipoxygenase-1 or 1.7 U of lipoxygenase-2 for 30 min at room temperature, by gently stirring in the presence of oxygen. Methanol (3 ml) was then added to inactivate the lipoxygenase, samples were vortexed and subjected to lipid extraction. Under the same experimental conditions 300 μM solutions of linoleic acid, α-linolenic acid or their methyl esters were incubated with lipoxygenase-2. In control experiments, biological membranes were incubated in the absence of lipoxygenase.

2.4. Lipid extraction and chromatographic analyses

Each sample of biomembranes (6 ml) was diluted with 6 ml MILLI-Q water and was loaded onto octadecyl-SPE columns (J.T. Baker). The free fatty acids and the esterified fatty acids present in each sample were eluted separately, by washing the columns with 8 ml methanol/water (50:50, v/v) and 6 ml methanol plus 4.5 ml ethyl acetate, respectively [28]. The recovery of the octadecyl-SPE cartridges, estimated by loading and eluting known amounts of 13-HOD, was quantitative.

The free fatty acid and esterified fatty acid fractions from membranes were reduced with $NaBH_4$. Then the esterified fatty acids were hydrolyzed in methanol/5 M KOH (4:1, v/v) for 30 min at 60°C under argon and the released free fatty acids were purified on the octadecyl-SPE columns with a mixture of methanol/water (50:50, v/v) as eluent. The free fatty acid samples were analyzed by RP-HPLC, performed on a HP1090 Liquid Chromatograph interfaced with a HP79994A Analytical Workstation (Hewlett Packard) and equipped with a Hewlett-Packard 1040A diode array detector. Samples were run on a CP-Spher C18 column (5 μm, 250 × 4.6 mm; Chrompack) and were isocratically eluted with tetrahydrofuran/methanol/water/acetic acid (25:30:45:0.1, v/v), at a flow rate of 1 ml/min. The chromatograms were recorded at 235 nm and 205 nm, in order to quantitate the oxidative modification of the biomembranes, as reported [28]. The amount of hydroxy fatty acids was calculated using the molar absorption coefficient at 235 nm of 25000 $M^{-1}cm^{-1}$ [5]. The identity of the compounds was ascertained by co-injection with authentic standards. The compounds were quantified by peak areas.

For the GC/MS analysis, the oxygenated products isolated from membranes incubated with lipoxygenase-2 were methylated by treatment with diazomethane for 10 min at room temperature. Then, the double bonds in the compounds were hydrogenated with hydrogen gas in the presence of a platinum catalyst (PtO_2, in methanol), for 30 min at room temperature. Finally, hydrogenated samples were converted into their trimethylsilyl ethers by the reaction with Sigma-Sil-A (Sigma) for 2 h at room temperature. Samples were eventually resuspended in n-hexane and subjected to GC/MS analysis on an apolar BP1 capillary column (0.5 μm phase thickness, 25 m × 0.33 mm internal diameter; SGE). GC conditions were isothermal for 2 min at 180°C, followed by temperature programming to 280°C at 5°C/min. The injector tempera-

ture was 300°C, the column pressure was 5 kPa and helium was used as carrier gas (2 ml/min flow). The column outlet was directly connected to the ionization source of a JEOL AX 505-W mass spectrometry system, operated at an ionization energy of 70 eV and kept at a temperature of 230°C.

Chiral phase HPLC was performed on naphthoyl derivatives of the hydroxy fatty acid methyl esters on a Bakerbond Chiral Phase™ (*R*)-*N*-3,5-dinitrobenzoyl phenylglycine column (covalently linked, 5 μm, 250 × 4.6 mm; J.T. Baker). Samples were derivatized and eluted as reported [29]. Analyses were performed on a Spectroflow 400 solvent delivery system coupled with a Spectroflow 783 uv detector (Kratos) and equipped with a Shimadzu C-R3A peak integrator. Isocratic elution was in n-hexane/2-propanol (100:0.25, v/v), at 0.8 ml/min flow rate.

The esterified fatty acids isolated from the mem-

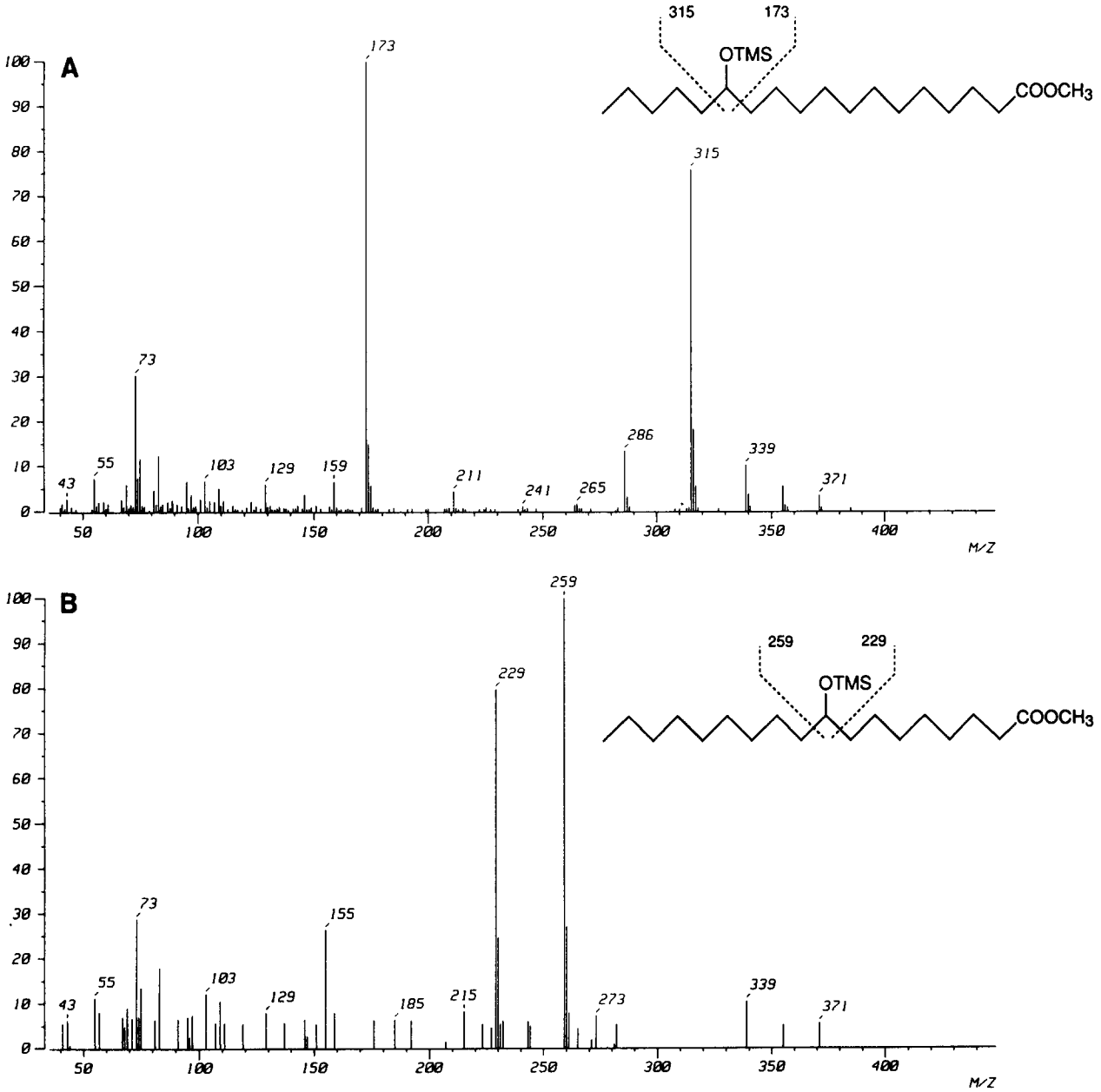

Fig. 1. RP-HPLC analysis of soybean membranes after alkaline hydrolysis. The biomembranes of soybean seedlings (3 mg of protein/ml) were incubated with either lipoxygenase-1 (3.4 U) or lipoxygenase-2 (1.7 U) for 30 min at room temperature, then lipids were extracted, separated in free fatty acid and esterified fatty acid fractions and reduced with $NaBH_4$. After alkaline hydrolysis of the esterified fatty acids, all fractions were analyzed by RP-HPLC. (A) Free fatty acid fraction incubated with either lipoxygenase-1 or -2. (B) Esterified fatty acid fraction incubated with lipoxygenase-2. The incubation of the ester lipids with the type-1 enzyme did not yield any product absorbing at 235 nm. Inset (A) Mass spectrum of 13-HOD, showing the expected peaks of the main fragmentation products at $m/z = 173$ and 315. Inset (B) Mass spectrum of 9-HOD, showing the diagnostic peaks at $m/z = 229$ and 259. 13-HOT and 9-HOT yielded the same mass spectra as 13-HOD and 9-HOD, respectively, because the double bonds of the compounds were hydrogenated before the GC/MS analysis.

brane pool were separated into different lipid classes by two dimensional HPTLC [30]. Phospholipids were identified by co-chromatography with authentic standards and were quantitated by assaying for total phosphorus content [31]. The amount of neutral lipids present in the esterified fatty acid fraction was estimated from the soybean membrane composition reported [32]. Lipids of the different classes were scraped off the plates, extracted [33] and resuspended in methanol, then their absorbances at 205 nm and 235 nm were recorded on a Hewlett Packard 8450A double-beam diode array spectrophotometer. Each data point reported in this paper is the mean of three independent determinations (S.D. $< 7\%$).

3. Results

3.1. Analysis of the oxygenated soybean membranes

After incubation of soybean biomembranes with lipoxygenases-1 or -2, total lipids were extracted. The free fatty acid and esterified fatty acid fractions were collected separately and treated with $NaBH_4$. The membrane free fatty acids and esterified fatty acids did not show any compound absorbing at 235 nm after incubation with lipoxygenase-1, yielding a chromatogram superimposable on that of the control sample (Fig. 1A). The type-2 enzyme was not able to oxygenate the membrane free fatty acids, but it did oxidize the esterified fatty acids in the lipid bilayers, yielding 13-HOT, 9-HOT, 13-HOD and 9-HOD after alkaline hydrolysis (Fig. 1B).

The identity of the oxygenation products absorbing at 235 nm was further investigated by GC/MS analysis, after fractionation followed by methylation, hydrogenation and silylation. The mass spectra showed the typical fragment ions at $m/z = 173$ and 315, expected from 13-HOD or 13-HOT (Fig. 1, inset A), and at $m/z = 229$ and 259, expected for 9-HOD or 9-HOT (Fig. 1, inset B). Thus, esterified linoleic and α-linolenic acids present in the biomembranes are the substrates for lipoxygenase-2.

The regio- and stereo-specificity of the products isolated from the biomembranes were compared with those of the products generated by lipoxygenase-2 from free linoleic and α-linolenic acids or their methyl esters. It appeared that the main HOD and HOT regioisomers isolated from membranes, i.e., 13-HOD and 9-HOT, had a higher stereospecificity compared to 13-HOD and 9-HOT originating from the free acids or their methyl esters. The values are reported in Table 1. The membrane products also had a different regiospecificity compared with that of the products generated from non-membraneous substrates, and showed a striking preference for 9-HOT and 13-HOD (Table 1). Furthermore, the specificity of the lipoxygenase-2 products was not significantly influenced by the meth-

Table 1
Regio- and stereospecificity of the soybean lipoxygenase-2 products

Substrate	13-HOT/ 9-HOT molar ratio	9*S*-/ 9*R*-HOT molar ratio	13-HOD/ 9-HOD molar ratio	13*S*-/ 13*R*-HOD molar ratio
Membrane pool	0.11	0.04	1.70	2.00
α-Linolenic acid	0.40	0.75		
Methyl linolenate	0.46	0.67		
Linoleic acid			0.38	1.22
Methyl linoleate			0.49	1.00

Lipoxygenase-2 (1.7 U) was incubated with the biomembranes isolated from soybean seedlings (3 mg of protein/ml) for 30 min at room temperature. The esterified fatty acids were extracted, hydrolyzed under alkaline conditions and analyzed by both RP-HPLC and chiral phase HPLC. α-Linolenic acid, methyl linolenate, linoleic acid and methyl linoleate were incubated under the same conditions as the biomembranes and were subjected to the same product analysis.

Table 2
Distribution of the hydroxy fatty acids in different lipid classes of soybean biomembranes

Lipid class	Amount of lipids (nmol/ml)	Hydroxy fatty acids (nmol/ml)	Hydroxy fatty acid/lipid molar ratio (%)	Hydroxy fatty acid/lipid relative ratio (%)	A_{235}/A_{205} ratio (%)	A_{235}/A_{205} relative ratio (%)
Neutral lipids	1780.00	253.6	14.2	100 [a]	9.7	100 [b]
Phosphatidylglycerol	140.47	10.0	7.1	50	4.7	48
Phosphatidylcholine	111.93	5.6	5.0	35	3.9	40
Pool of PX [c]	82.80	4.0	4.8	34	3.0	31
Phosphatidylinositol	28.00	2.4	8.6	60	5.4	56
Phosphatidylethanolamine	24.53	2.0	8.1	57	5.0	52

The biomembranes of soybean seedlings were incubated with lipoxygenase-2 as reported in Table 1, then the esterified fatty acids were extracted and analyzed by means of two-dimensional HPTLC. The different lipid classes were identified and quantitated as described under Materials and methods. The amount of hydroxy fatty acids produced by the lipoxygenase-2 activity was related to the amount of total lipids present in each class.

[a] The hydroxy fatty acid/lipid ratio of the neutral lipid fraction was arbitrarily set at 100.

[b] The A_{235}/A_{205} ratio of the neutral lipid fraction was arbitrarily set at 100.

[c] PX indicates unidentified phospholipids.

Table 3
Distribution of the oxygenation products of lipoxygenase-2 in different membrane fractions

Subcellular fraction	HOT/HOD molar ratio (%)	13-HOT/9-HOT molar ratio (%)	13-HOD/9-HOD molar ratio (%)	A_{235}/A_{205} relative ratio (%)
Chloroplasts	1.7	1.94	0.75	100 [a]
Mitochondria	11.9	0.39	1.44	30
Endopl. reticulum	13.9	0.18	1.63	62
Plasma membr.	8.9	0.30	1.22	33
Golgi apparatus	3.1	0.30	1.57	66
Membrane pool	14.6	0.11	1.70	67

Cell organelles were isolated from soybean seedlings and were incubated with lipoxygenase-2 as described in Table 1. Lipids were extracted, the esterified fatty acids were reduced under alkaline conditions and analyzed by RP-HPLC, recording the absorbances at 205 nm and 235 nm.

[a] The A_{235}/A_{205} ratio of the chloroplast lipids was arbitrarily set at 100.

ylation of the substrates (Table 1).

The distribution of hydroperoxy fatty acids in different lipid classes was investigated, by separating the lipids of the membrane pool by means of two dimensional HPTLC. In Table 2 it is shown that neutral lipids and phospholipids contained hydroperoxy fatty acids, the highest amount being detected in the former fraction. The same trend was observed when expressing the extent of oxygenation of the lipid classes by means of the ratio A_{235}/A_{205} (Table 2). Therefore, this ratio was confirmed to be a suitable parameter to express the oxidative modification of lipids [28].

3.2. Subcellular fractions

After isolating the subcellular fractions, these were incubated with lipoxygenase-2 and the oxygenation products were analyzed. The RP-HPLC profiles of the esterified fatty acids, obtained after reduction and alkaline hydrolysis resulted in the quantitative data summarized in Table 3. All subcellular fractions, with the exception of chloroplasts, showed product specificities similar to each other and to that of the membrane pool, HOT being preferentially formed as regioisomer 9-HOT and HOD as regioisomer 13-HOD. On the other hand, the differences in HOT/HOD ratios might be a consequence of different contents of α-linolenic and linoleic acid residues in the various membranes. Unlike the other subcellular fractions, chloroplast membranes yielded 13-HOT and 9-HOD as main oxygenation products. Moreover, chloroplast membranes were oxidized by lipoxygenase-2 to a greater extent than the other fractions, as indicated by the A_{235}/A_{205} ratio (Table 3). Control incubations of the organelles' membranes in the absence of the type-2 enzyme yielded chromatograms without peaks absorbing at 235 nm.

4. Discussion

Here, we show that soybean lipoxygenase-2, unlike the type-1 enzyme, oxygenates plant biological membranes (Fig. 1). The observation that the ester lipids present in the biomembranes were a substrate for lipoxygenase-2, at variance with the free fatty acid fraction (Fig. 1), is in agreement with the preference for uncharged substrates shown by the enzyme in solution [5,34]. Interestingly, both linoleic and α-linolenic residues present in the biomembranes were attacked by lipoxygenase-2 in a way different from the free fatty acids in solution. The membrane products exhibited a higher degree of stereospecificity and a striking preference for the regioisomer 13-HOD over 9-HOD (Table 1). This finding points to a clear difference between the reaction catalyzed by lipoxygenase-2 towards a complex substrate like biomembranes and the reaction occurring with solubilized substrates, where low product specificities are typically observed [35]. Furthermore, the remarkable changes in product specificity of soybean membranes represent an important difference compared to the mammalian system, where the same product specificity was observed using free fatty acids, solubilized phospholipids or biomembranes as substrates [28,36]. Soybean lipoxygenase-2 was able to oxidize both neutral lipids and phospholipids of membrane bilayers (Table 2). The ratio of free fatty acids to esterified fatty acids in soybean biomembranes is reported to be 0.3% [32]. Neutral lipids, representing the major component of soybean seedling biomembranes [32], were oxygenated to a larger extent, corroborating previous observations on the lipoxygenase-mediated oxidation of neutral esters in solution [37]. This finding is interesting in the light of the role of the neutral lipids in stabilizing the molecular architecture of soybean membranes [38]. The various phospholipids contained similar amounts of hydroperoxy fatty acids, suggesting that the different polar head groups had little effect on the suitability of the phospholipids as substrates of lipoxygenase-2.

Lipoxygenase-2 is active towards both intracellular and plasma membranes. Chloroplasts, which contain approximately 10% of the soybean membranes [27], were oxidized to a larger extent by lipoxygenase-2. This is noteworthy, because the enzyme has been proposed to control the chloroplast biogenesis [39] and, moreover, its expression is light-regulated [11]. Nevertheless, the ability of lipoxygenase-2 to oxygenate various cellular membranes is in favour of a general role for the enzyme in regulating or triggering different processes in the cell, rather than being primarily involved in any particular organelle-related process. The intracellular localization of the enzyme, showing that nearly every subcellular compartment is a site of lipoxygenase [40], corroborates the hypothesis. It is, therefore, tempting

to suggest that in vivo a type-2 lipoxygenase might modulate the properties of biomembranes through lipid peroxidation [41]. This could represent a common feature in many different physiological processes where lipoxygenase is involved.

In conclusion, evidence is presented that soybean lipoxygenase-2 can oxygenate esterified fatty acid residues with high regio- and stereo-specificity in biomembranes. These findings call for some reappraisal of the features of the reaction catalyzed by the lipoxygenase-2, which in vivo might be very different from an "enzyme-catalyzed autoxidation" of unsaturated fatty acids as observed for the conversion of unsaturated fatty acids in solution around pH 7.0. The physicochemical features of lipid bilayers responsible for the changes in product specificity observed in this study are under investigation, with the aim of elucidating the elements of the membrane architecture which might modulate the lipoxygenase-2 reaction.

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