Enzymic characterization of epidermis-derived 12-lipoxygenase isoenzymes

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Substrate selectivity and other enzymic characteristics of two epidermis-derived lipoxygenases (LOXs), the epidermis-type (*e*) (12*S*)-LOX and (12*R*)-LOX, were compared with those of the platelet-type (*p*) (12*S*)-LOX. In contrast with *p*(12*S*)-LOX, *e*(12*S*)-LOX and (12*R*)-LOX exhibited no or very low reactivity towards the customary substrates linoleic acid and arachidonic acid but metabolized the corresponding fatty acid methyl esters, which, in contrast, were not accepted as substrates by *p*(12*S*)-LOX. Other esters of arachidonic acid and linoleic acid, including propan-2-yl and cholesterol esters, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-linoleyl-*sn*-glycero-3-phosphoethanolamine, and ceramide 1 carrying an ω-linoleic acid ester, were not metabolized by these three LOX isoenzymes. Among various polyunsaturated fatty acids the isomeric eicosatrienoic acids were found to be oxygenated by *e*(12*S*)-LOX but not by (12*R*)-LOX. 4,7,10,13,16,19-Docosahexaenoic acid as a substrate was restricted to *p*(12*S*)-LOX. Variations in the pH and the Ca^{2+} content of the incubation medium affected the catalytic potential only slightly. Whereas (12*R*)-LOX activity increased in the presence of Ca^{2+} and with an acidic pH, Ca^{2+} had no effect on *p*(12*S*)-LOX and *e*(12*S*)-LOX; an acidic pH decreased the catalytic activity of the latter two. However, the catalytic activity of the epidermis-type isoenzymes, but not of *p*(12*S*)-LOX, was found to be markedly increased in the presence of DMSO. Under these conditions, *e*(12*S*)-LOX and (12*R*)-LOX oxygenated 4,7,10,13,16,19-docosahexaenoic acid to 14-hydroxy-4,7,10,12,16,19-docosahexaenoic acid and 13-hydroxy-4,7,10,14,16,19-docosahexaenoic acid respectively. In addition, (9*R*)-hydroxyoctadeca-10,12-dienoic acid methyl ester was generated from linoleic acid methyl ester by (12*R*)-LOX. Independently of the substrate, the catalytic activity of *e*(12*S*)-LOX and (12*R*)-LOX was always at most 2% of that of *p*(12*S*)-LOX with arachidonic acid as substrate.

Key words: dioxygenation, polyunsaturated fatty acids, (12*R*)-lipoxygenase, (12*S*)-lipoxygenase.

INTRODUCTION

In addition to 'conventional' lipoxygenases (LOXs) such as (5*S*)-LOX, leucocyte-type (*l*) and platelet-type (*p*) (12*S*)-LOX, and reticulocyte-type (15*S*)-LOX-1, the mammalian LOX family of dioxygenases has recently expanded to comprise several isoenzymes that were found to be preferentially expressed in the epidermis of man and mice. They include novel LOX enzymes such as the epidermis-type (*e*) (12*S*)-LOX and (12*R*)-LOX, the mouse (8*S*)-LOX and its human orthologue (15*S*)-LOX-2, and *e*-LOX-3, a mouse LOX with an as yet unknown enzymic activity. These novel isoenzymes can be regarded as a distinct epidermis-type subgroup of the LOX multigene family (reviewed in [1,2]).

The 'conventional' LOX enzymes prefer arachidonic acid as a substrate, which is oxygenated to regioisomeric hydroperoxy-eicosatetraenoic acids and, on reduction, to the corresponding hydroxyeicosatetraenoic acids (HETEs). They also accept linoleic acid but with varying activity. This is in sharp contrast with the epidermis-type LOXs, which exhibit only very low or even no catalytic activity with both arachidonic and linoleic acids when tested as recombinant enzymes [3–12]. It is possible that efficient catalytic activity depends on post-translational modifications that are not provided by expression of the recombinant proteins in non-keratinocyte cell lines. Furthermore, arachidonic acid and linoleic acid might not be appropriate substrates for the epidermis-type LOX. In fact, we recently have shown that mouse (12*R*)-LOX converted methyl arachidonate but not the non-esterified fatty acid into the corresponding (12*R*)-HETE ester [5]. The low catalytic activity of the recombinant epidermis-type LOX might also be due to the incubation conditions used in the cell-free activity assays previously developed for 'conventional' LOX enzymes.

In this study we have characterized the enzymic properties of *e*(12*S*)-LOX and (12*R*)-LOX in comparison with those of *p*(12*S*)-LOX with regard to substrate and assay conditions. Our results strengthen the notion that non-esterified arachidonic acid and linoleic acid are not the native substrates of the epidermis-type LOX.

MATERIALS AND METHODS

Materials

Arachidonic acid and linoleic acid, and also the corresponding methyl esters, 5,8,11-eicosatrienoic acid, 8,11,14-eicosatrienoic acid, 4,7,10,13,16,19-docosahexaenoic acid, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-linoleyl-sn-glycero-3-phosphoethanolamine, were from Sigma (München, Germany). (8*S*)- and (8*R*)-hydroxyeicosa-(5*Z*,9*E*,11*Z*,14*Z*)-tetraenoic acid [(8*S*)-HETE and (8*R*)-HETE], (12*S*)- and (12*R*)-hydroxyeicosa-(5*Z*,8*Z*,10*E*,14*Z*)-tetraenoic acid [(12*S*)-HETE and (12*R*)-HETE)], (9*S*)- and (9*R*)-hydroxyoctadeca-(10*E*,12*Z*)-dienoic acid [(9*S*)-HODE and (9*R*)-HODE)], (13*S*)- and (13*R*)-hydroxyoctadeca-(9*Z*,11*E*)-dienoic

Abbreviations used: *e*, epidermis-type; ESI, electrospray ionization; HDHE, hydroxydocosahexaenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; *l*, leucocyte-type; LOX, lipoxygenase; *p*, platelet-type.

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[(13*S*)-HODE and (13*R*)-HODE)] standards were from Reatec (Weiterstadt, Germany). Methyl ester standards were prepared from the corresponding acids by methylation with diazomethane. All other chemicals and solvents were of analytical grade.

Expression of LOX cDNA

For transient expression, plasmid DNA (10 μg) and vector DNA as control were introduced into HEK-293 cells (seeded at 1.4×10^6 cells per 100 mm plate) with a modified calcium phosphate transfection procedure by using a mammalian transfection kit in accordance with the manufacturer's protocol (Stratagene). Cells were harvested 48 h after transfection. When recombinant LOXs were compared for their catalytic activity, they were obtained from the same series of transfection experiments. The efficiency of every set of transfections was determined by introducing a β-galactosidase expression plasmid into HEK-293 cells and counting β-galactosidase-positive cells or assaying β-galactosidase activity in cell lysates. The mean transfection efficiency was found to be $54 \pm 10\,\%$ ($n \geqslant 50$).

Preparation of 10000 *g* supernatants

Transfected cells were homogenized by sonication (Branson Sonifier, 10 pulses within 10 s on ice) in TEE buffer [50 mM Tris/HCl (pH 7.4)/1 mM EDTA/1 mM EGTA/10 μg/ml leupeptin] or 50 mM sodium phosphate buffer, pH 7.4, 6.6 or 6.0, containing 1 mM EDTA and 1 mM EGTA. The supernatant obtained after centrifugation (10000 ***g*** for 10 min at 4 °C) was used for the LOX assay.

LOX assay

Aliquots of the 10000 ***g*** supernatants containing 50 μg of protein for *p*(12*S*)-LOX or 800 μg of protein for *e*(12*S*)-LOX and (12*R*)-LOX were diluted to 250 μl with the appropriate sample buffer (see above) and incubated with 100 μM of arachidonic acid, linoleic acid, arachidonic acid methyl ester, linoleic acid methyl ester, 5,8,11-eicosatrienoic acid, 8,11,14-eicosatrienoic acid or 4,7,10,13,16,19-docosahexaenoic acid, each dissolved in 5 μl of ethanol, for 12 min at 37 °C. Incubations in the presence of DMSO were performed by evaporating ethanol and resolubilizing the residue with various amounts of DMSO after vortex mixing. Incubations in the presence of Ca^{2+} were performed by adding 2.1 mM $CaCl_2$, yielding a final free Ca^{2+} concentration of 0.2 mM (see below). The incubations were terminated by adding 40 μl of 1 M sodium formate buffer, pH 3.1. To reduce the hydroperoxy acids to hydroxy acids, 20 μl of trimethylphosphite was added and incubated at room temperature for 5 min [13]. Samples were extracted twice with ethyl acetate. The combined organic extracts were evaporated and analysed by reverse-phase HPLC. Individual products were collected and subjected to straight-phase and/or chiral-phase chromatography or analysed by MS (see below).

Calculation of free Ca^{2+} concentration

Total Ca^{2+} and Mg^{2+} concentrations in cell preparations were determined as being 110 and 830 μM respectively, by using inductively coupled plasma mass spectroscopy ('ICPMS'). Binding constants for EDTA and EGTA were calculated by the method of Blinks et al. [14] and Durham [15] as being 10^8 for Ca^{2+}/EDTA and $10^{7.7}$ for Ca^{2+}/EGTA. Binding constants for Mg^{2+} were lower by factors of 32 and 2×10^5 respectively. Thus the concentration of free Ca^{2+} in the reaction mixture was calculated as 10 pM and, after the addition of $CaCl_2$, as 200 μM.

Reverse-phase and straight-phase HPLC analysis of HETEs and HODEs and the corresponding methyl esters

Reverse-phase HPLC analysis of HETE, HODE and the corresponding methyl esters was performed with a YMC-Pack ODS-AM column (25 cm × 0.46 cm, 5 μm particle size; YMC Europe, Schermbeck, Germany) with a 1 cm guard column. Products were eluted at 0.5 ml/min with a solvent system consisting of methanol/water/acetic acid (86:14:0.01, by vol.). Elution was monitored at 236 and 205 nm. The products were identified and quantified by comparing retention times and peak areas with those of authentic external standards. Retention times were 17.4 min for 13-HODE, 17.6 min for 9-HODE, 18.2 min for 13-hydroxydocosahexaenoic acid (13-HDHE), 18.7 min for 14-HDHE, 19.8 min for 12-HETE, 20.3 min for 8-HETE, 22.7 min for 12-hydroxyeicosa-8,10,14-trienoic acid, 25.3 min for 12-hydroxyeicosa-5,8,10-trienoic acid, 25.6 min for 13-HODE methyl ester, 26.0 min for 9-HODE methyl ester, 28.1 min for 12-HETE methyl ester and 29.0 min for 8-HETE methyl ester.

Straight-phase HPLC analysis of HODE methyl ester fractions from reverse-phase HPLC was performed by using a Zorbax Sil column (25 cm × 0.46 cm, 5 μM particle size; Bischoff, Stuttgart, Germany). Products were eluted at 1.0 ml/min with a solvent system consisting of n-hexane/propan-2-ol/water/acetic acid (98.5:1.5:0.025/0.1, by vol.). Elution was monitored at 236 and 205 nm. The products were identified and quantified by comparing retention times and peak areas with those of authentic external standards. Retention times were 8.5 min for 13-HODE methyl ester and 9.9 min for 9-HODE methyl ester.

Chiral-phase analysis of HETEs and HODEs and the corresponding methyl esters

Fractions of HETEs, HODEs, and the corresponding methyl esters obtained from the reverse-phase HPLC were rechromatographed by chiral-phase HPLC using a Chiracel OB column (25 cm × 0.46 cm; 5 μm particle size; Baker Instruments Corp., Deerfield, IL, U.S.A.) eluted at 0.5 ml/min with the solvent system n-hexane/propan-2-ol/acetic acid in various proportions: 97.5:2.5:0.05 (by vol.) for 9-HODE, 13-HODE, 8-HETE and 8-HETE methyl ester, 99:1:0.05 (by vol.) for 12-HETE and 12-HETE methyl ester and 95:5:0.05 (by vol.) for 9-HODE methyl ester and 13-HODE methyl ester. The products were identified and quantified by comparing retention times and peak areas with those of authentic external standards. The eluates were monitored at 236 nm. Retention times were 29.5 min for (9*S*)-HODE, 50.0 min for (9*R*)-HODE, 18.0 min for (13*S*)-HODE, 21.9 min for (13*R*)-HODE, 21.4 min for (8*S*)-HETE, 28.4 min for (8*R*)-HETE, 20.5 min for (8*S*)-HETE methyl ester, 26.8 min for (8*R*)-HETE methyl ester, 26.0 min for (12*S*)-HETE, 20.6 for (12*R*)-HETE, 33.8 min for (12*S*)-HETE methyl ester, 30.3 min for (12*R*)-HETE methyl ester, 17.2 min for (9*S*)-HODE methyl ester, 14.0 min for (9*R*)-HODE methyl ester, 16.4 min for (13*S*)-HODE methyl ester and 14.7 min for (13*R*)-HODE methyl ester.

Electrospray ionization (ESI) tandem MS

Mass spectra were recorded with a triple-quadrupole instrument type TSQ 7000 (Thermoquest, San Jose, CA, U.S.A.) equipped with a nanoESI source (EMBL, Heidelberg, Germany). Spray capillaries were prepared in-house with a micropipette puller

(Sutter Instruments) and were subsequently surface-coated with a semitransparent film of gold by using a gold sputter unit. The spray voltage used was 400–700 V; argon was used as the collision gas at a pressure of 0.267 Pa. Product fractions obtained from reverse-phase HPLC were evaporated to dryness and the residues were dissolved in methanol/water (4:1, v/v). Aliquots of approx. 5 μl were transferred into nanoESI capillaries. Each capillary was used for a single experiment only. For recording of product-ion spectra, 20–50 consecutive scans of 5 s duration were averaged.

RESULTS

To compare the enzymic activity of mouse *e*(12*S*)-LOX and (12*R*)-LOX with that of the 'conventional' *p*(12*S*)-LOX, the recombinant proteins were transiently expressed in HEK-293 cells by using the eukaryotic expression vector pcDNA3, which carries the corresponding cDNA species under the control of a cytomegalovirus promoter. LOX preparations used for comparing catalytic effects were obtained by transfecting a series of HEK-293 cultures seeded, cultivated and processed under identical conditions. When incubated with saturating concentrations of the substrates for 12 min, i.e. during the linear phase of the enzyme reaction, linear relationships between product formation and the sample's protein concentration of up to 0.2 mg/ml for *p*(12*S*)-LOX and up to 3.2 mg/ml for *e*(12*S*)-LOX and 12(*R*)-LOX were observed. Contents and profiles of oxygenated fatty acids in samples of vector-transfected cultures did not show significant variations within the same or between different transfection series. In contrast with HEK-293 cells, recombinant expression in mouse or human keratinocytes did not yield amounts of 12-LOX proteins sufficient for enzymic analysis.

Arachidonic acid was metabolized to (12*S*)-HETE and linoleic acid to (13*S*)-HODE as main products by *e*(12*S*)-LOX and *p*(12*S*)-LOX, although the catalytic potential of *e*(12*S*)-LOX with these substrates was only approx. 0.1 % and 6 % respectively of that of *p*(12*S*)-LOX (Table 1) [3–5,10–12]. Mouse (12*R*)-LOX did not accept the non-esterified fatty acids as substrates. Instead,

Table 1 Specific enzymic activities and reaction products of recombinant mouse *e*(12*S*)-LOX, (12*R*)-LOX and *p*(12*S*)-LOX with arachidonic acid, linoleic acid and the corresponding methyl esters as substrates

Supernatants (10000 ***g***) from sonicated HEK-293 cells transfected with *p*(12*S*)-LOX (0.2 mg/ml protein), *e*(12*S*)-LOX and (12*R*)-LOX (3.2 mg/ml protein) or pcDNA3 vector alone (3.2 mg/ml protein) were incubated for 12 min at 37 °C in TEE buffer with 100 μM arachidonic acid, linoleic acid and the corresponding methyl esters. The hydroperoxy derivatives were reduced and extracted twice with ethyl acetate. Reverse-phase and chiral-phase chromatography and product-specific retention times are described in the Materials and methods section. Quantities given in parentheses were measured by comparing the peak areas of the products with those of corresponding external standards. The amounts of HETEs, HODEs and methyl esters generated by vector-transfected cells were subtracted. Results are means ± S.E.M. for $n \geqslant 3$ independent experiments. Abbreviation: n.a., no activity.

		Reaction products and quantities (pmol/mg of protein)		
Substrate	Enzyme ...	*p*(12*S*)-LOX	*e*(12*S*)-LOX	(12*R*)-LOX
Arachidonic acid		(12*S*)-HETE (194590 ± 6538) (8*R*)-HETE (560 ± 19)	(12*S*)-HETE (95 ± 6)	n.a.
Linoleic acid		(13*S*)-HODE (3515 ± 1310)	(13*S*)-HODE (205 ± 70)	n.a.
Arachidonic acid methyl ester		n.a.*	(12*S*)-HETE methyl ester (180 ± 18)	(12*R*)-HETE methyl ester (180 ± 107) (8*R*)-HETE methyl ester (55 ± 28)
Linoleic acid methyl ester		n.a.*	(13*S*)-HODE methyl ester (1325 ± 468)	n.a.

* (12*S*)-HETE and (13*S*)-HODE formed through substrate hydrolysis (see Figure 2C) are not shown.

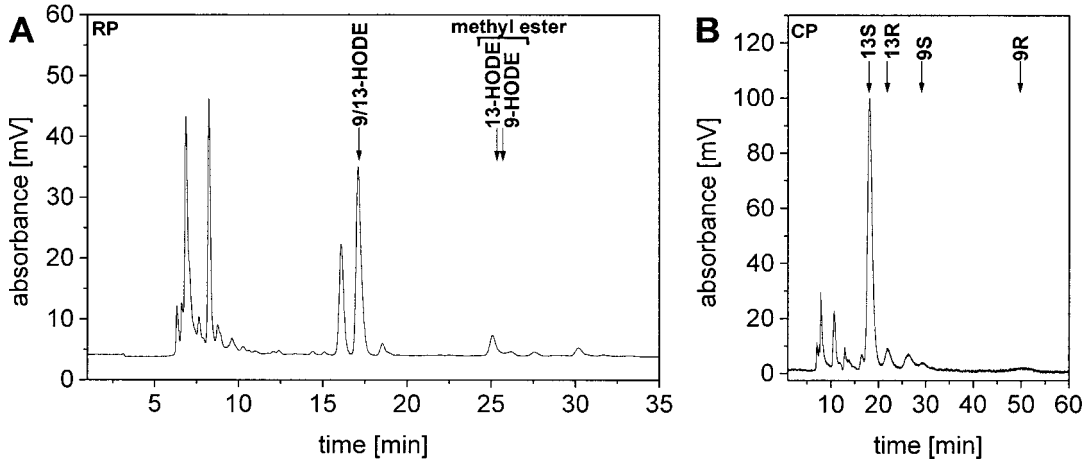


Figure 1 Oxygenation of linoleic acid methyl ester by recombinant *e*(12*S*)-LOX

Supernatants (10000 ***g***) of HEK-293 cells transfected with an *e*(12*S*)-LOX expression plasmid were incubated for 12 min at 37 °C in TEE buffer with 100 μM linoleic acid methyl ester. The hydroperoxy derivatives were reduced, extracted with ethyl acetate and analysed by reverse-phase HPLC. 13-HODE and 13-HODE methyl ester fractions were collected and subjected to chiral-phase chromatography as described in the Materials and methods section. (**A**) Reverse-phase HPLC of the ethyl acetate extract; (**B**) chiral-phase chromatography of the 13-HODE fraction.

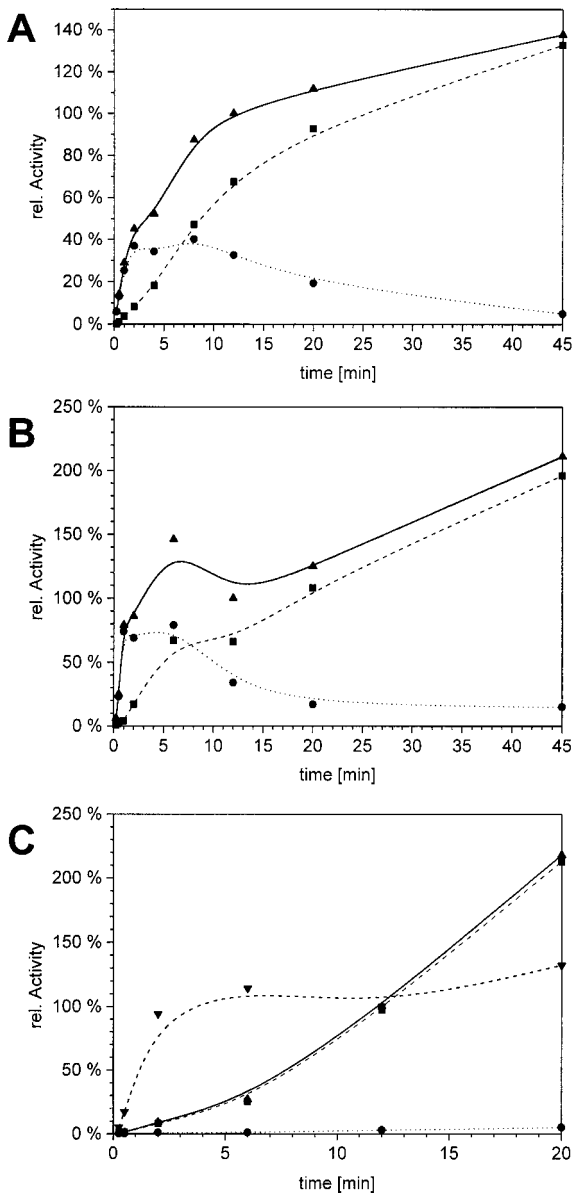


Figure 2 Kinetics of substrate oxygenation by (12*R*)-LOX, *e* (12*S*)-LOX and *p*(12*S*)-LOX

Supernatants (10000 ***g***) of HEK-293 cells transfected with (12*R*)-LOX, *e*(12*S*)-LOX or *p*(12*S*)-LOX expression plasmids were incubated for various durations at 37 °C in TEE buffer with 100 μM arachidonic acid methyl ester, linoleic acid methyl ester and non-esterified arachidonic acid respectively. The hydroperoxy derivatives were reduced, extracted with ethyl acetate and analysed by reverse-phase HPLC as described in the Materials and methods section. Activity is shown as a percentage of the amount of products formed after a 12 min incubation period [100% = 235 pmol/mg of protein for (12*R*)-LOX, 1325 pmol/mg of protein for *e*(12*S*)-LOX, and 194590 pmol/mg of protein for *p*(12*S*)-LOX]. (**A**) (12*R*)-LOX incubated with arachidonic acid methyl ester; (**B**) *e*(12S)-LOX incubated with linoleic acid methyl ester; (**C**) *p*(12*S*)-LOX incubated with arachidonic acid and arachidonic acid methyl ester. Results are one representative set out of three independent experiments. Symbols, ■, non-esterified HETE/HODE; ●, HETE/HODE methyl ester; ▲, total product (sum of non-esterified and methylated HETE/HODE); ▼, total product formation from arachidonic acid.

it oxygenated arachidonic acid methyl ester to (12*R*)-HETE methyl ester and (8*R*)-HETE methyl ester, albeit with rather low efficiency. Arachidonic acid methyl ester was also a substrate of *e*(12*S*)-LOX, yielding (12*S*)-HETE methyl ester with approximately twice the efficiency of the non-esterified acid (Table 1). *e*(12*S*)-LOX also oxygenated linoleic acid methyl ester to (13*S*)-HODE methyl ester, thereby exhibiting an approx. 6-fold higher activity than with non-esterified linoleic acid. In contrast, (12*R*)-LOX did not accept methyl linoleate; both methyl linoleate and methyl arachidonate were essentially not substrates of *p*(12*S*)-LOX (Table 1 and Figure 1). Other esters of linoleic acid and arachidonic acid, including propan-1-yl and cholesterol esters, ceramide 1 carrying an ω-linoleic acid ester, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-linoleyl-*sn*-glycero-3-phosphoethanolamine, were inactive as substrates for the *e*(12*S*)-LOX and (12*R*)-LOX and for *p*(12*S*)-LOX (results not shown).

On incubation of methyl arachidonate or methyl linoleate with (12*R*)-LOX and *e*(12*S*)-LOX, the corresponding non-esterified fatty acids (12*R*)-HETE and (13*S*)-HODE were also formed (Figures 2A and 2B). This was due to secondary hydrolysis, probably by an esterase activity present in the 10000 ***g*** supernatants from transfected and non-transfected HEK-293 cells. In fact, the kinetic analyses of product formation shown in Figures 2(A) and 2(B) documented the methyl esters of (12*R*)-HETE and (13*S*)-HODE as being the primary products that were subsequently hydrolysed to the corresponding non-esterified fatty acids. In contrast, (12*S*)-HETE formation seen on the incubation of *p*(12*S*)-LOX with arachidonic acid methyl ester clearly depended on prior methyl ester hydrolysis, showing that the non-esterified fatty acid rather than the methyl ester was the substrate for this isoenzyme (Figure 2C).

In another set of experiments, several polyunsaturated fatty acids known to occur in epidermis or to exhibit biological effects when administered to skin [16] were tested as potential substrates of *e*(12*S*)-LOX and (12*R*)-LOX in comparison with *p*(12*S*)-LOX. The latter enzyme and *e*(12*S*)-LOX, although with significantly lower efficiency, oxygenated 5,8,11-eicosatrienoic acid to 12-hydroxy-5,8,10-eicosatrienoic acid, and 8,11,14-eicosatrienoic acid to 12-hydroxy-8,10,14-eicosatrienoic acid (Table 2). The identities of the isomeric hydroxyeicosatrienoic acids were assigned by ESI MS/MS analysis yielding position-specific fragmentation patterns (Table 3). (12*R*)-LOX did not accept the eicosatrienoic acids as substrates (Table 2). 4,7,10,13,16,19-Docosahexaenoic acid was oxygenated by *p*(12*S*)-LOX, yielding 14-hydroxy-4,7,10,12,16,19-hexaenoic acid, whereas *e*(12*S*)-LOX and (12*R*)-LOX were inactive under these incubation conditions.

The catalytic capacity of the enzymes individually depended on the pH and the Ca^{2+} concentration of the incubation medium. Thus, 200 μM Ca^{2+} increased the activity of (12*R*)-LOX but had essentially no effect on *p*(12*S*)-LOX and *e*(12*S*)-LOX (Figure 3). Decreasing the pH from 7.4 to 6.0 decreased the catalytic activity of *p*(12*S*)-LOX and *e*(12*S*)-LOX but slightly increased that of (12*R*)-LOX. In addition, when DMSO was used instead of ethanol as a solvent, the catalytic activities of (12*R*)-LOX with arachidonic acid methyl ester and of *e*(12*S*)-LOX with linoleic acid methyl ester as substrates were found to be increased. The stimulatory effect of DMSO was dose-dependent, reaching a maximum at approx. 20% (Figures 4A and 4B). In contrast, DMSO was without effect in incubations of arachidonic acid with *p*(12*S*)-LOX (Figure 4C). DMSO not only increased the catalytic activity of mouse *e*(12*S*)-LOX and (12*R*)-LOX with linoleic or arachidonic acid methyl ester but also allowed other substrates to be oxygenated. Thus, only in the presence of DMSO was (12*R*)-LOX found to oxygenate linoleic acid methyl ester to the corresponding (9*R*)-HODE methyl ester (Table 4). Moreover, non-esterified 4,7,10,13,16,19-docosahexaenoic acid was metabolized by (12*R*)-LOX to 13-hydroxy-4,7,10,14,16,19-hexaenoic acid and by *e*(12*S*)-LOX to

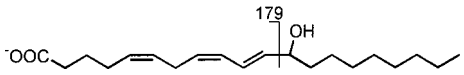
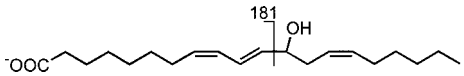
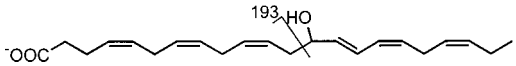
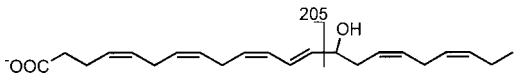
Table 2 Substrate specificity for mouse *e*(12*S*)-LOX, (12*R*)-LOX and *p*(12*S*)-LOX

Supernatants (10000 ***g***) from sonicated HEK-293 cells transfected with *p*(12*S*)-LOX (0.2 mg/ml protein), *e*(12*S*)-LOX and (12*R*)-LOX (3.2 mg/ml protein each) or pcDNA3 vector alone (3.2 mg/ml protein) were incubated for 12 min at 37 °C in TEE buffer with 100 μM arachidonic acid, 5,8,11-eicosatrienoic acid, 8,11,14-eicosatrienoic acid, and 4,7,10,13,16,19-docosahexaenoic acid. The hydroperoxy derivatives were reduced and extracted twice with ethyl acetate. Reverse-phase and chiral-phase chromatography and product-specific retention times are described in the Materials and methods section. Activities given in parentheses were measured by comparing the peak areas of the products with those of corresponding external standards. The amounts of HETEs, HODEs and methyl esters generated by vector-transfected cells were subtracted. Results are means $\pm$ S.E.M. for $n \geqslant 3$ independent experiments. Abbreviations: n.a., no activity; 12-HE(5,8,10)TrE, 12-hydroxy-5,8,10-eicosatrienoic acid; 12-HE(8,10,14)TrE, 12-hydroxy-8,10,14-eicosatrienoic acid; 14-HDHE, 14-hydroxy-4,7,10,12,16,19-docosahexaenoic acid.

		Main reaction products and quantities (pmol/mg of protein)		
Substrate	Enzyme ...	*p*(12*S*)-LOX	*e*(12*S*)-LOX	(12*R*)-LOX
Arachidonic acid		(12*S*)-HETE (194590 $\pm$ 6538)	(12*S*)-HETE (95 $\pm$ 6)	n.a.
5,8,11-Eicosatrienoic acid		12-HE(5,8,10)TrE (2635 $\pm$ 312)	12-HE(5,8,10)TrE (170 $\pm$ 107)	n.a.
8,11,14-Eicosatrienoic acid		12-HE(8,10,14)TrE (31680 $\pm$ 13506)	12-HE(8,10,14)TrE (755 $\pm$ 68)	n.a.
4,7,10,13,16,19-Docosahexaenoic acid		14-HDHE (33955 $\pm$ 12598)	n.a.	n.a.

Table 3 ESI-MS/MS analysis of monohydroxy unsaturated fatty acids

Supernatants (10000 ***g***) from sonicated HEK-293 cells transfected with *p*(12*S*)-LOX (0.2 mg/ml protein), *e*(12*S*)-Lox and (12*R*)–LOX (3.2 mg/ml protein each) or pcDNA3 vector alone (3.2 mg/ml protein) were incubated for 12 min at 37 °C in TEE buffer with 100 μM 5,8,11-eicosatrienoic acid, 8,11,14-eicosatrienoic acid and (in the presence of DMSO) 4,7,10,13,16,19-docosahexaenoic acid. The hydroperoxy derivatives were reduced and extracted twice with ethyl acetate. Reverse-phase chromatography and product-specific retention times are described in the Materials and methods section. Product fractions obtained from reverse-phase HPLC were evaporated to dryness and the residues were dissolved in methanol/water (4:1, v/v). Aliquots of approx. 5 μl were subjected to ESI-MS/MS. The hydroxyl substituent position was derived from key fragments obtained by MS/MS of the respective molecular ions by the method described in [17].

Compound	Molecular ion (*m*)	Key fragments	
12-Hydroxyeicosa-5,8,11-trienoic acid	321	$[A^-]$	
	303	$[A^- - H_2O]$	
	277	$[A^- - CO_2]$	
	259	$[A^- - H_2O - CO_2]$	
	179	α-Fragmentation	
12-Hydroxyeicosa-8,11,14-trienoic acid	321	$[A^-]$	
	303	$[A^- - H_2O]$	
	277	$[A^- - CO_2]$	
	259	$[A^- - H_2O - CO_2]$	
	181	α-Fragmentation	
13-Hydroxydocosa-4,7,10,14,16,19-hexaenoic acid	343	$[A^-]$	
	325	$[A^- - H_2O]$	
	299	$[A^- - CO_2]$	
	281	$[A^- - H_2O - CO_2]$	
	193	α-Fragmentation	
14-Hydroxydocosa-4,7,10,12,16,19-hexaenoic acid	343	$[A^-]$	
	325	$[A^- - H_2O]$	
	299	$[A^- - CO_2]$	
	281	$[A^- - H_2O - CO_2]$	
	205	α-Fragmentation	

14-hydroxy-4,7,10,12,16,19-hexaenoic acid. The structures of the regioisomeric products were derived from their characteristic product-ion spectra (Table 3) [17].

DISCUSSION

Mouse skin is known to express three 12-LOX isoenzymes constitutively. These include *p*(12*S*)-LOX, which has also been detected in other cell types and tissues, and *e*(12*S*)-LOX and (12*R*)-LOX, which have been found to be expressed predominantly in the integumental epithelium of mice [3,5,6,8,18]. As shown here, these structurally diverse enzymes differ strongly in their enzymic activities and substrate specificities.

The substrate fatty acids tested comprised, in addition to linoleic acid and arachidonic acid, C_{20} and C_{22} polyunsaturated fatty acids known to be present or to exhibit physiological activities in skin epidermis, namely two isomeric eicosatrienoic acids and ($\omega-3$)-docosahexaenoic acid [16]. None of the non-esterified fatty acids tested was found to be a substrate of mouse (12*R*)-LOX in conditions under which human (12*R*)-LOX has been shown to metabolize non-esterified arachidonic acid, 8,11,14-eicosatrienoic acid and 4,7,10,13,16,19-docosahexaenoic acid, although with very low efficiency [8,19]. Only in the presence of DMSO did mouse (12*R*)-LOX metabolize 4,7,10,13,16,19-docosahexaenoic acid (but not arachidonic acid and linoleic acid), generating 13-hydroxy-4,7,10,12,16,19-docosahexaenoic acid, whereas the human analogue has been reported to produce 14-hydroxy-4,7,10,13,15,19-docosahexaenoic acid [19]. The latter was also the predominant product obtained with *p*(12*S*)-LOX [20] and with *e*(12*S*)-LOX, as shown here. Whereas

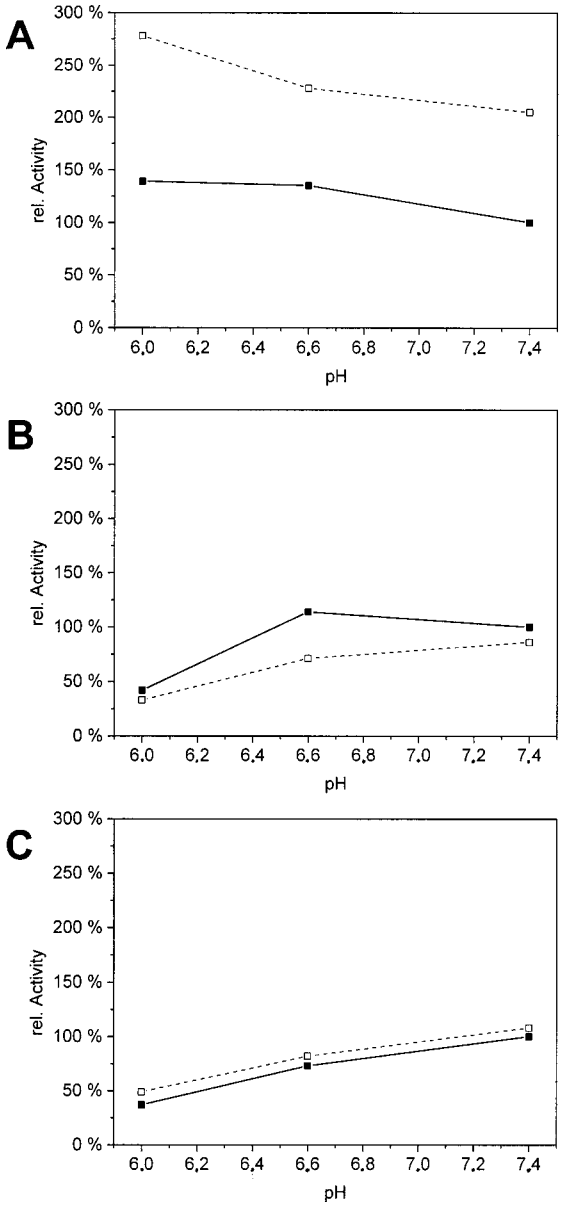


Figure 3 Effects of Ca^{2+} and pH on the catalytic activity of (12*R*)-LOX, *e*(12*S*)-LOX and *p*(12*S*)-LOX

Supernatants (10 000 ***g***) of HEK-293 cells transfected with (12*R*)-LOX, *e*(12*S*)-LOX or *p*(12*S*)-LOX expression plasmids were incubated for 12 min at 37 °C in TEE buffer with 100 μM arachidonic acid methyl ester, linoleic acid methyl ester and non-esterified arachidonic acid respectively. The hydroperoxy derivatives were reduced, extracted with ethyl acetate and analysed by reverse-phase HPLC as described in the Materials and methods section. Activity is shown as a percentage of the amount of products formed at pH 7.4 in the absence of Ca^{2+} [100% = 235 pmol/mg of protein for (12*R*)-LOX, 1325 pmol/mg of protein for *e*(12*S*)-LOX, and 194 590 pmol/mg of protein for *p*(12*S*)-LOX]. (**A**) (12*R*)-LOX incubated with arachidonic acid methyl ester; (**B**) *e*(12*S*)-LOX incubated with linoleic acid methyl ester; (**C**) *p*(12*S*)-LOX incubated with arachidonic acid. Results are one representative set out of three independent experiments. Symbols: ■, incubation in the absence of Ca^{2+}; □, incubation in the presence of 0.2 mM Ca^{2+}.

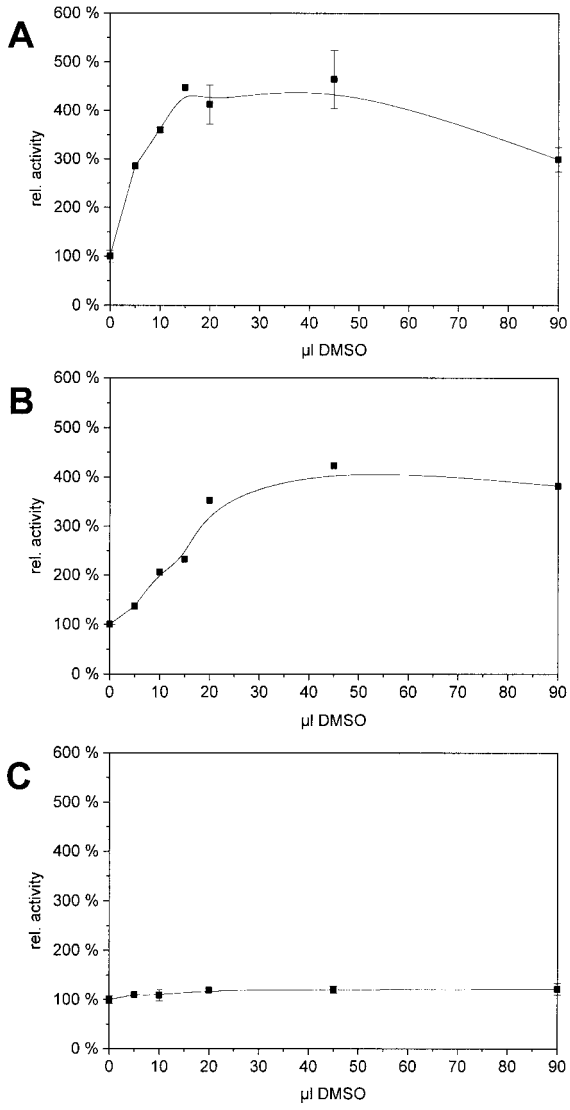


Figure 4 Effects of DMSO on the catalytic activity of (12*R*)-LOX, *e*(12*S*)-LOX or *p*(12*S*)-LOX

Supernatants (10 000 ***g***) of HEK-293 cells transfected with (12*R*)-LOX, *e*(12*S*)-LOX or *p*(12*S*)-LOX expression plasmids were incubated for 12 min at 37 °C in TEE buffer with 100 μM arachidonic acid methyl ester, linoleic acid methyl ester and non-esterified arachidonic acid respectively, dissolved in 5 μl of ethanol or in increasing amounts of DMSO (up to 90 μl). The hydroperoxy derivatives were reduced and extracted with ethyl acetate and analysed by reverse-phase HPLC as described in the Materials and methods section. Activity is shown as a percentage of the amount of products formed in the absence of DMSO (100% = 235 pmol/mg of protein for (12*R*)-LOX, 1325 pmol/mg of protein for *e*(12*S*)-LOX, and 194 590 pmol/mg of protein for *p*(12*S*)-LOX). (**A**) (12*R*)-LOX incubated with arachidonic acid methyl ester; (**B**) *e*(12*S*)-LOX incubated with linoleic acid methyl ester; (**C**) *p*(12*S*)-LOX incubated with arachidonic acid. Results are one representative set out of three independent experiments.

hydroperoxidation at position 14 of docosahexaenoic acid by human (12*R*)-LOX is thought to be initiated through hydrogen abstraction at C-12, formation of the 13-hydroperoxy derivative by the mouse analogue requires the removal of a hydrogen from C-15 [21]. The reason for these species differences is not yet clear.

The *e*(12*S*)-LOX showed increasing catalytic activity within the series arachidonic acid < 5,8,11-eicosatrienoic acid < linoleic acid < 8,11,14-eicosatrienoic acid. However, the maximum activity was less than 7% of that of *p*(12*S*)-LOX.

A striking feature of *e*(12*S*)-LOX and (12*R*)-LOX was their reactivity towards methyl esters of linoleic and arachidonic acids, whereas *p*(12*S*)-LOX was completely inactive in this regard. (13*S*)-HODE methyl ester and (9*R*)-HODE methyl ester were

Table 4 Substrate specificity for *e*(12*S*)-LOX, (12*R*)-LOX and *p*(12*S*)-LOX: effect of DMSO

Supernatants (10000 ***g***) from sonicated HEK-293 cells transfected with *p*(12*S*)-LOX (0.2 mg/ml protein), *e*(12*S*)-LOX and (12*R*)-LOX (3.2 mg/ml protein each) or pcDNA3 vector alone (3.2 mg/ml protein) were incubated for 12 min at 37 °C in TEE buffer with 100 μM arachidonic acid, linoleic acid and the corresponding methyl esters, and also 4,7,10,13,16,19-docosahexaenoic acid, dissolved in 5 μl of DMSO. The hydroperoxy derivatives were reduced and extracted twice with ethyl acetate. Reverse-phase and chiral-phase chromatography and product-specific retention times are described in the Materials and methods section. Quantities given in parentheses were measured by comparing the peak areas of the products with those of corresponding external standards. The amounts of HETEs, HODEs and methyl esters generated by vector-transfected cells were subtracted. Results are means $\pm$ S.E.M. for $n \geqslant 3$ independent experiments. Abbreviations: n.d., not determined; n.a., no activity; 14-HDHE, 14-hydroxy-4,7,10,12,16,19-docosahexaenoic acid; 13-HDHE, 13-hydroxy-4,7,10,14,16,19-docosahexaenoic acid.

		Main reaction products and quantities (pmol/mg of protein)		
Substrate	Enzyme ...	*p*(12*S*)-LOX	*e*(12*S*)-LOX	(12*R*)-LOX
Arachidonic acid		(12*S*)-HETE (207650 $\pm$ 30588)	n.d.	n.a.
Linoleic acid		n.d.	n.d.	n.a.
Arachidonic acid methyl ester		n.d.	n.d.	(12*R*)-HETE methyl ester (875 $\pm$ 469)
Linoleic acid methyl ester		n.d.	(13*S*)-HODE methyl ester (1990 $\pm$ 698)	(9*R*)-HODE methyl ester (240 $\pm$ 22)
4,7,10,13,16,19-Docosahexaenoic acid		n.d.	14-HDHE (920 $\pm$ 89)	13-HDHE (205 $\pm$ 21)

the respective major products of the *e*(12*S*)-LOX and (12*R*)-LOX reaction with linoleic acid methyl ester. Methyl esters of (12*R*)-HETE and (8*R*)-HETE were formed from arachidonic acid methyl ester as pure enantiomers by (12*R*)-LOX-catalysed oxygenation. It has previously been shown [8] that synthesis of (12*R*)-HETE by human (12*R*)-LOX is associated with removal of the pro-*R* hydrogen from C-10 of arachidonic acid and oxygen insertion into the antarafacial (12*R*)-position. Oxygen insertion at the mirror-image isomeric 8-position would then be expected to yield (8*S*)-HETE. However, the 8-HETE methyl ester obtained with mouse (12R)-LOX was clearly the (8*R*)-enantiomer. According to the well established 'antarafacial oxygen insertion rule', (8*R*)-HETE methyl ester formation must be preceded by removal of the pro-*S* hydrogen at C-10 of methyl arachidonate [22]. In fact, (8*R*)-HETE was found to be generated as a side-product of arachidonic acid oxygenation by *p*(12*S*)-LOX, which is known to remove this pro-*S* hydrogen [12]. Thus the alternative removal of the prochiral hydrogens at C-10 might be involved in the generation of (12*R*)-HETE methyl ester and (8*R*)-HETE methyl ester. Provided that the catalytic centres of (12*S*)-LOX and (12*R*)-LOX are structurally related, removal of the pro-*R* hydrogen at C-10 and subsequent formation of (12*R*)-HETE methyl ester is conceivable with an inverse orientation of the substrate, i.e. arachidonic acid methyl ester penetrating the binding cleft with its carboxy ester group [22]. This arrangement forces a charged group into an essentially hydrophobic surrounding area and might thus lead to an impairment of LOX-catalysed oxygenation. This has indeed been shown for dicarboxylic acid derivatives of linoleic acid used as substrates of (15*S*)-LOX-1. The introduction of an ω-carboxy group led to a marked decrease in the catalytic activity that could be partly restored by methylation of the carboxy group [23]. Thus the inverse orientation would also explain the lack of detectable activity of the mouse (12*R*)-LOX towards non-esterified arachidonic acid and linoleic acid and the low activity towards the methyl esters. Formation of (8*R*)-HETE methyl ester would then be conceivable with the 'normal' orientation of the methyl ester, i.e. with the methyl end penetrating into the substrate-binding cavity. From the low yield of the (8*R*) product one can conclude that this substrate orientation is not favourable for a (12*R*)-LOX reaction. Thus the simultaneous generation of (12*R*)-HETE methyl ester and (8*R*)-HETE methyl ester would be plausible only by tolerating both the 'normal' and the inverse orientations of arachidonic acid methyl ester [12].

The identification of linoleic and arachidonic acid methyl esters as artificial substrates of *e*(12*S*)-LOX and (12*R*)-LOX might be taken as a hint of the nature of endogenous substrates, which could be esterified polyunsaturated fatty acids. This is not without precedent, because both (15*S*)-LOX-1 and *l*(12*S*)-LOX have previously been shown to oxygenate phospholipid-bound arachidonic acid (reviewed in [2]). However, this notion was not supported by the results of this study, because none of the other ester species thought to be potential substrates were metabolized by the recombinant enzymes.

A decrease in pH of the incubation medium slightly increased the catalytic activity of mouse (12*R*)-LOX towards arachidonic acid methyl ester, but strongly decreased those of *e*(12*S*)-LOX and *p*(12*S*)-LOX towards linoleic acid methyl ester and arachidonic acid respectively. No change in the product spectrum was observed. In contrast, a decrease in pH in the incubation medium was reported to result in a strong increase in the catalytic activity of human (12*R*)-LOX towards arachidonic acid [24]. The mouse isoenzyme did not show such a response. The presence of free Ca^{2+} in the incubation medium significantly increased the catalytic efficiency of (12*R*)-LOX but remained essentially without effect on that of *e*(12*S*)-LOX and *p*(12*S*)-LOX. Ca^{2+} has been shown to be involved in the association of platelet-type (12*S*)-LOX and (15*S*)-LOX-1 with membranes, thereby increasing the enzymic activity towards membrane lipids and non-esterified polyunsaturated fatty acids [25,26]. Whether or not this also holds true for (12*R*)-LOX remains to be shown. In this context it is remarkable that the expression of this enzyme is restricted to suprabasal cell layers of epidermis [18], where the cellular Ca^{2+} concentration is known to be markedly increased over that found in keratinocytes of the basal compartment (reviewed in [27]).

Replacement of ethanol by DMSO as solvent of substrates did not change the catalytic activity of *p*(12*S*)-LOX but distinctly improved that of *e*(12*S*)-LOX and (12*R*)-LOX. The mechanism of this DMSO effect is currently a matter of speculation. DMSO might increase the solubility of the substrate [28], thus yielding a threshold concentration necessary for the activity of *e*(12S)-LOX and (12R)-LOX, an effect that might not be required for the much more active 'conventional' enzymes. DMSO might also be able to increase LOX activity by modulating the physical properties of phospholipids present in 10000 ***g*** supernatants. Indeed, phospholipids have been shown to associate with and increase the activity of (5*S*)-LOX and (15*S*)-LOX-1 [26,29]. Finally, a direct stimulatory effect of the solvent on the enzymes cannot be excluded. In fact, DMSO up to 30% (v/v) has been shown to increase the activity of seminal vesicle cyclo-oxygenase and 12-LOX [30].

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