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Cationic substrates of soybean lipoxygenase-1

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ABSTRACT

Soybean lipoxygenase-1 (SBLO-1) catalyzes the oxygenation of 1,4-dienes to produce conjugated diene hydroperoxides. The best substrates are anions of fatty acids; for example, linoleate is converted to 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate. The manner in which SBLO-1 binds substrates is uncertain. In the present work, it was found that SBLO-1 will oxygenate linoleyltrimethylammonium ion (LTMA) to give primarily13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienyltrimethylammonium ion. The rate of this process is about the same at pH 7 and pH 9 and is about 30% of the rate observed with linoleate at pH 9. At pH 7, SBLO-1 oxygenates linoleyldimethylamine (LDMA) to give primarily 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienyldimethylamine. The oxygenation of LDMA occurs at about the same rate as LTMA at pH 7, but more slowly at pH 9. The results demonstrate that SBLO-1 will readily oxygenate substrates in which the carboxylate of linoleate is replaced with a cationic group, and the products of these reactions have the same stereo- and regiochemistry as the products obtained from fatty acid substrates.

1. Introduction

Lipoxygenases catalyze the formation of hydroperoxides from the 1,4-diene units of polyunsaturated fatty acids and derivatives [1,2]. In plants, lipoxygenases are involved in storage-lipid mobilization and in the synthesis of jasmonic acid and other oxylipins [3]. In animals, lipoxygenases catalyze key steps in the synthesis of leukotrienes and other inflammatory mediators [4] and appear to be involved in membrane modification during cell differentiation and modification [1,5]. Inhibitors of human lipoxygenases are of interest as potential drugs for asthma [6], inflammation [4], atherosclerosis [7] and cancer [8].

The most extensively studied lipoxygenase is soybean lipoxygenase-1 (SBLO-1). This enzyme catalyzes the oxygenation of linoleate to 13-(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate (13-HPOD, Scheme 1). SBLO-1 will also oxygenate other fatty acids [9], phospholipids [10–12], and various other substrates that have a 1,4-diene unit that begins in the ω6 position of an alkyl chain [13,14]. Like almost all lipoxygenases, SBLO-1 is a non-heme iron protein, and its catalytic mechanism appears to involve redox cycling of the iron between the ferrous and ferric states [15,16]. The iron is located in the center of a large helical domain [17,18]. Crystal structures are available for several other plant lipoxygenases [19,20], a lipoxygenase from coral [21], and one mammalian lipoxygenase [22,23], and these enzymes have very similar secondary and tertiary structures to SBLO-1.

An important unanswered question about SBLO-1 and other lipoxygenases is the manner in which substrates bind. Answering this question is necessary in order to fully understand how these enzymes control the stereo- and regiochemistry of oxygenation [24] and would also aid in the design of lipoxygenase inhibitors. An additional challenge for SBLO-1 and mammalian 15-lipoxygenase is to account for the fact that these enzymes will oxygenate not only free fatty acids but also larger substrates such as phospholipids [10–12,25].

It has been known for some time that SBLO-1 will oxygenate fatty acid anions more readily than neutral substrates such as methyl linoleate. In considering possible modes of substrate binding, we became interested in whether SBLO-1 would oxygenate substances in which the carboxylate group of linoleate was replaced by a positively charged group. In 1977, Bild et al. [13] reported that oxygen uptake was observed when SBLO-1 was incubated with 10,13-nonadecadienamine and noted that the amino group of this substrate was probably positively charged under the conditions of their experiments. Interestingly, the rate of oxygen uptake was higher than observed with most neutral substrates. The interpretation of this result is uncertain, because the products of the reaction were not identified. In this paper, we report the synthesis of linoleyldimethylamine (LDMA, Scheme 2) and

Abbreviations: BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; EIMS, electron-impact mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; GC/MS, gas chromatography/mass spectrometry; 13-HOD, 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoate; 13-HPOD, 13-(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate; LDMA, linoleyldimethylamine; LTMA, linoleyltrimethylammonium ion; NMR, nuclear magnetic resonance; SBLO-1, soybean lipoxygenase-1; TLC, thin layer chromatography.

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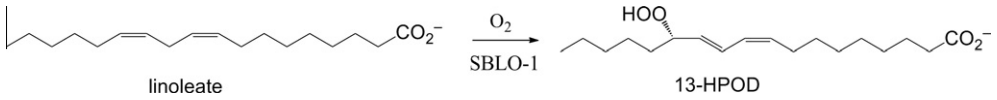
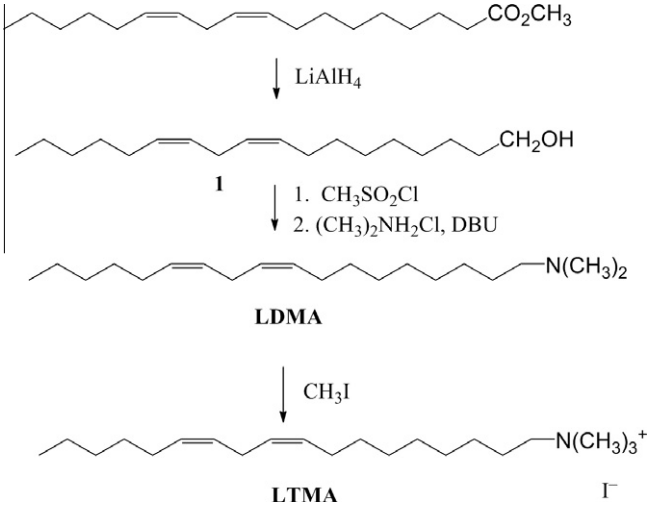


Scheme 1. Reaction catalyzed by SBLO-1.



Scheme 2. Synthesis of LDMA and LTMA.

linoleyltrimethylammonium iodide (LTMA) and demonstrate that these substances undergo oxygenation by SBLO-1 to give products analogous to those obtained with fatty acid substrates.

2. Materials and methods

2.1. Synthesis

The synthesis of LDMA and LTMA is outlined in Scheme 2, and the procedures are described below. Methyl linoleate (Sigma/Aldrich) was reduced to linoleyl alcohol (**1**) by a published method [26]. NMR spectra were obtained on a Varian 400 MHz spectrometer or a Bruker ARX 300 MHz instrument, and chemical shifts are reported relative to tetramethylsilane.

2.1.1. Linoleyldimethylamine (LDMA)

Methanesulfonyl chloride (7.1 mmol, 0.81 g) was added dropwise at 0 °C to a stirred solution of 1.49 g (5.6 mmol) of linoleyl alcohol (**1**) and 0.85 g (8.4 mmol) of triethylamine. The mixture was stirred for 45 min at 0 °C and 45 min at room temperature. The reaction mixture was washed with 20-mL portions of water, 10% HCl, 10% $NaHCO_3$, and 10% NaCl, and the organic layer was concentrated to an oil, which gave a single spot by TLC ($CHCl_3$/MeOH/H_2O (64:25:4) R_f = 0.86) and had a ^{1}H NMR spectrum consistent with the methanesulfonate of linoleyl alcohol: (400 MHz, $CDCl_3$) δ 5.35 (4H, m), 4.22 (2H, t, J = 6.6 Hz), 3.00 (3H, s), 2.77 (2H, t, J = 6.4 Hz), 2.05 (4H, q, J = 7 Hz), 1.75 (2H, quintet, J = 7 Hz), 1.30 (16H, m), 0.89 (3H, t, J = 6.9 Hz). A solution of 0.94 g (2.7 mmol) of the methanesulfonate, 7.23 g (88.7 mmol) of dimethylamine hydrochloride and 1.00 g (6.6 mmol) of 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU) in 12 mL of methanol was heated in a pressure tube at 56 °C for 48 h. The methanol was removed on a rotary evaporator, and the residue was partitioned between 70 mL of ether and 70 mL of 5% K_2CO_3. The organic layer was washed with two 25-mL portions of 1 M NaCl, dried with $MgSO_4$, and concentrated on a rotary evaporator. LDMA can be purified by flash chromatography on silica gel 60 with methanol/ethyl acetate/acetic acid (55:45:2). This procedure yields the acetate salt of LDMA, which can be converted to the free base by treatment with 5 M NaOH followed by extraction with dichloromethane and concentration to an oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.36 (4H, m), 2.77 (2H, t, J = 6.5 Hz), 2.25 (2H, m), 2.22 (6H, s), 2.05 (4H, q, J = 6.7 Hz), 1.45 (2H, m), 1.29 (16H, m), 0.89 (3H, t, J = 6.8 Hz). EIMS: m/z 293 (M^+).

2.1.2. Linoleyltrimethylammonium iodide (LTMA)

A solution of 275 mg (0.94 mmol) of LDMA and 773 mg (6.7 mmol) of methyl iodide in 15 mL of acetone was stirred for 20 h at room temperature. Concentration *in vacuo* yielded an amorphous gum. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (4H, m), 3.60 (2H, m), 3.47 (9H, s), 2.77 (2H, t, J = 6.3 Hz), 2.04 (4H, q, J = 6.7 Hz), 1.77 (2H, m), 1.26 (16H, m), 0.89 (3H, t, J = 6.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 130.5, 130.2, 128.3, 128.1, 67.5, 54.0, 31.7, 29.8, 29.6, 29.5, 29.4, 29.3, 27.4, 27.4, 26.3, 25.7, 23.4, 22.8, 14.3. ESI-MS: m/z 309 (M+). TLC [$CHCl_3$/MeOH/H_2O (64:25:4)] R_f = 0.32.

2.2. Enzymes and assays

SBLO-1 was purified from soybeans by the procedure of Axelrod et al. [27], and its concentration was determined spectrophotometrically using $A^{0.1}_{280nm} = 1.6$ [28]. One unit of lipoxygenase activity is the amount of enzyme that will convert linoleic acid to 13-HPOD at a rate of 1.0 μmole/min under the conditions described previously [29].

Oxygen consumption was measured polarographically with a Clarke-type electrode in a Hansatech D.W. Oxygen Electrode Unit maintained at 25 °C with a circulating water bath. Reactions were initiated by addition of enzyme (2.5–10 μL) to solutions of substrate in either 50 mM potassium phosphate, pH 7.0, or 50 mM borate, pH 9.0, in a total volume of 0.50 mL.

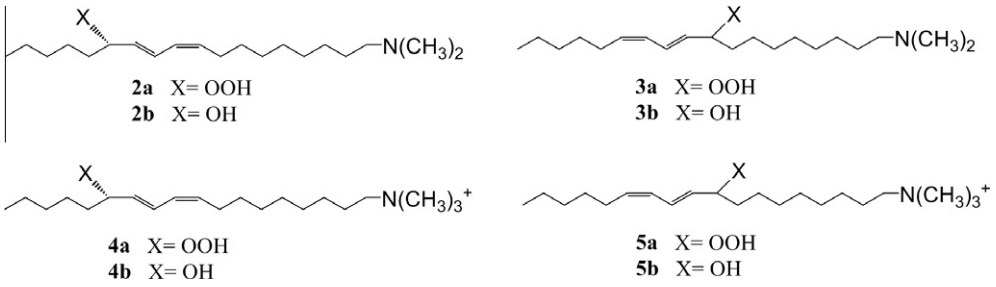
Peroxide was determined by the ferrous/xylenol orange method [30] or by using a coupled enzyme assay with glutathione peroxidase and glutathione reductase [31], which were obtained from Sigma/Aldrich.

2.3. Mass spectrometry

GC/MS analyses were carried out on a Hewlett–Packard GCD instrument with a 12 m × 0.3 mm HPI capillary column. The column temperature was maintained at 50 °C for 3 min and then increased from 50 to 250 °C at 20°/min. Electrospray MS and MS/MS experiments were carried out on a Sciex API III$^+$ triple-quadrupole instrument operated in the positive ion mode at an ionization voltage of 3 kV. Samples were infused into the capillary in 3 mM ammonium formate in methanol/water (1:1). MS/MS experiments were carried out with argon/nitrogen (9:1) as collision gas and a collision energy of 30 eV.

2.4. Lipoxygenase-catalyzed oxygenation of LDMA

A 200-mL solution of 0.18 mM LDMA in 50 mM phosphate buffer, pH 7.0, was treated with 20 units of SBLO-1, and the mixture was stirred vigorously at room temperature and monitored by measuring the A_{234} of 1:10 dilutions of aliquots withdrawn periodically. After 30 min, the A_{234} leveled off (at 0.26 in a 1:10 dilution),



Scheme 3. Hydroperoxides and alcohols derived from LDMA and LTMA.

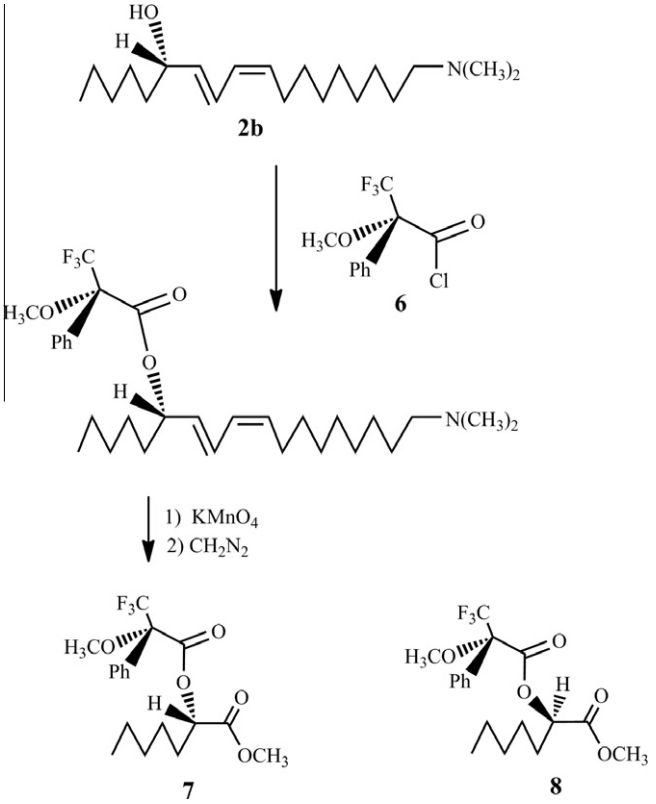
and the reaction mixture was extracted with ether (3 × 60 mL). Concentration of the extracts yielded an oil, which was dissolved in 3 mL of ethanol and treated with 26 mg of $NaBH_4$ at 0 °C. After 30 min of stirring, the excess $NaBH_4$ was destroyed by cautious addition of acetic acid (*ca* 1 mL), and the mixture was extracted with ether (3 × 10 mL). Concentration of the extracts yielded an oil, which will be referred to as the reduced product from LDMA. This material gave a single spot (R_f = 0.26) by TLC in hexanes/methanol (98:2) and a ^{1}H NMR spectrum that contained the resonances expected for either **2b** or **3b** (Scheme 3): (300 MHz, $CDCl_3$): δ 6.49 (1H, dd, J = 15.1, 11.1 Hz), 5.97 (1H, t, J = 10.9 Hz), 5.67 (1H, dd, J = 15.1, 6.8 Hz), 5.42 (1H, dt, J = 10.8, 7.6 Hz), 4.15 (1H, q, J = 6.6 Hz), 2.53 (2H, m), 2.44 (6H, s), 2.18 (2H, q, J = 7.2 Hz). Approximately 1 mg of this substance in 1.0 mL of methanol was stirred under H_2 for 1 h with 10 mg of 5% Pd on $CaCO_3$. The catalyst was removed by centrifugation, and the methanol was evaporated under a stream of nitrogen. The residue was incubated with 10 μL of pyridine and 10 μL of BSTFA for 1 h at room temp. to convert the hydroxyl group to its trimethylsilyl derivative for GC/MS analysis.

2.5. Methylation of reduced product from LDMA

A 3.5-mg portion of the reduced product from LDMA was stirred with 15 mg of methyl iodide in 4 mL of acetone for 8 h at room temperature. An additional 40 mg of methyl iodide was added, and the mixture was stirred for another 16 h. At this point, TLC ($CHCl_3$/CH_3OH/H_2O, 65:25:4) indicated complete conversion to a product with R_f = 0.66. This material was used for the MS/MS spectrum in Fig. 4B.

2.6. Lipoxygenase-catalyzed oxygenation of LTMA

To a stirred solution of 20 mg of LTMA in 150 mL of 25 mM $(NH_4)_2CO_3$, pH 9.0, was added 6 units SBLO-1. After 35 min the A_{234} in a 1:10 dilution had risen to 0.74. The reaction mixture was concentrated on a rotary evaporator with a mechanical vacuum pump. The residue was dissolved in $CDCl_3$, and residual buffer salts were removed by centrifugation. The ^{1}H NMR spectrum (400 MHz) contained the signals expected for **4a** and/or **5a**: δ 6.42 (1H, dd, J = 15.2, 11.1 Hz), 5.91 (1H, t, J = 11.0 Hz), 5.60 (1H, dd J = 15.1, 6.7 Hz), 5.36 (1H, dt, J = 10.7, 7.6 Hz), 4.10 (q, J = 6.5 Hz), 3.51 (m), 3.31 (s). Signals for unreacted LTMA were also present. About 5 mg of this material in 1.0 mL of ethanol was treated with $NaBH_4$ (10 mg), and the mixture was stirred for 30 min at 0 °C and 30 min at room temperature. The $NaBH_4$ was quenched with glacial acetic acid (~100 μL), and the reaction mixture was loaded onto a 20 × 1 cm column of Al_2O_3. The column was eluted with ethanol, and the portion of the eluent that absorbed at 234 nm was concentrated. The residue gave an NMR spectrum similar to that obtained before $NaBH_4$ treatment. This material was also analyzed by electrospray MS and MS/MS (Fig. 4A).



Scheme 4. Stereochemical analysis.

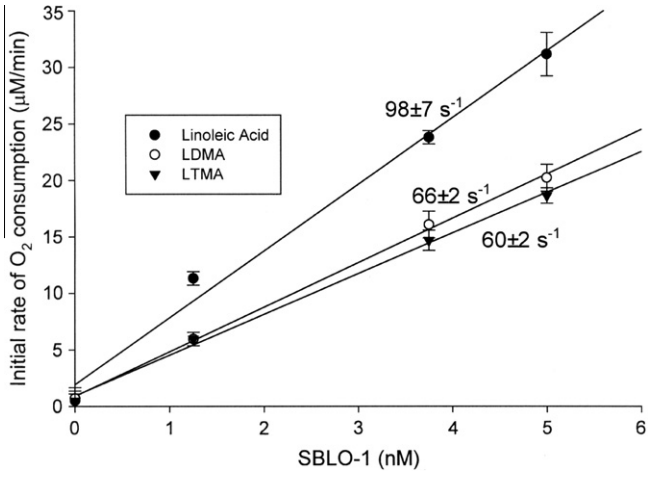


Fig. 1. Initial rates of oxygenation of linoleic acid, LDMA and LTMA by SBLO-1 at pH 7.0, 25 °C. Each reaction contained 72 μM of the indicated substrate. The slopes are indicated next to trend lines.

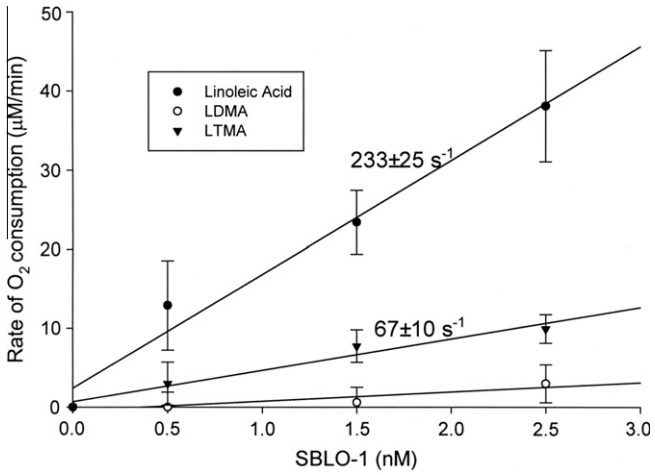


Fig. 2. Initial rates of oxygenation of linoleic acid, LTMA and LDMA by SBLO-1 at pH 9.0, 25 °C. Each reaction contained 72 μM of the indicated substrate. The slopes are indicated next to trend lines.

2.7. Stereochemical analysis

Six milligrams of the reduced product from LDMA was treated with 59 μL of pyridine, 18 μL of methylene chloride and 6 μL of (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (**6**, Scheme 4), which was obtained from Sigma/Aldrich. After 5 h at room temp, the reaction mixture was concentrated under N_2 and the product was purified by preparative TLC (hexanes/ethyl acetate/acetic acid 7:3:1; R_f = 0.60). This material was dissolved in 300 μL of acetic acid and treated with 22 mg of solid $KMnO_4$. The mixture was incubated for 2 h at 37 °C and then diluted with 1 mL of water and extracted with ether (2 × 0.5 mL). The ether extracts were washed with water (1.0 mL) and concentrated to dryness. The residue was methylated with diazomethane to give **7** and/or **8**. A synthetic mixture of **7** and **8** was prepared from **6** and racemic methyl 2-hydroxyheptanoate, which was prepared by a published method [32]. Stereochemical analysis of the product from LTMA was carried out in the same manner, except that the TLC purification of the product from the first step was omitted.

3. Results

Fig. 1 shows the rates of O_2 consumption that occurred when increasing concentrations of SBLO-1 were incubated at 25 °C with 72 μM solutions of LDMA, LTMA or linoleic acid in 50 mM potassium phosphate buffer, pH 7.0. The data indicate that LDMA and LTMA undergo enzymatic oxygenation at about equal rates, which are about 65% of the rate observed with linoleic acid. Fig. 2 shows the results of a similar experiment, with the same concentration of substrates, carried out in 50 mM borate buffer, pH 9.0. Linoleic acid is a considerably better substrate for SBLO-1 at pH 9.0 than at pH 7.0 [13,33], and this gives rise to a 2.4-fold increase in the slope in the plot of rate vs enzyme when linoleic acid is the substrate. (Numerical values for the slopes are displayed next to the trend

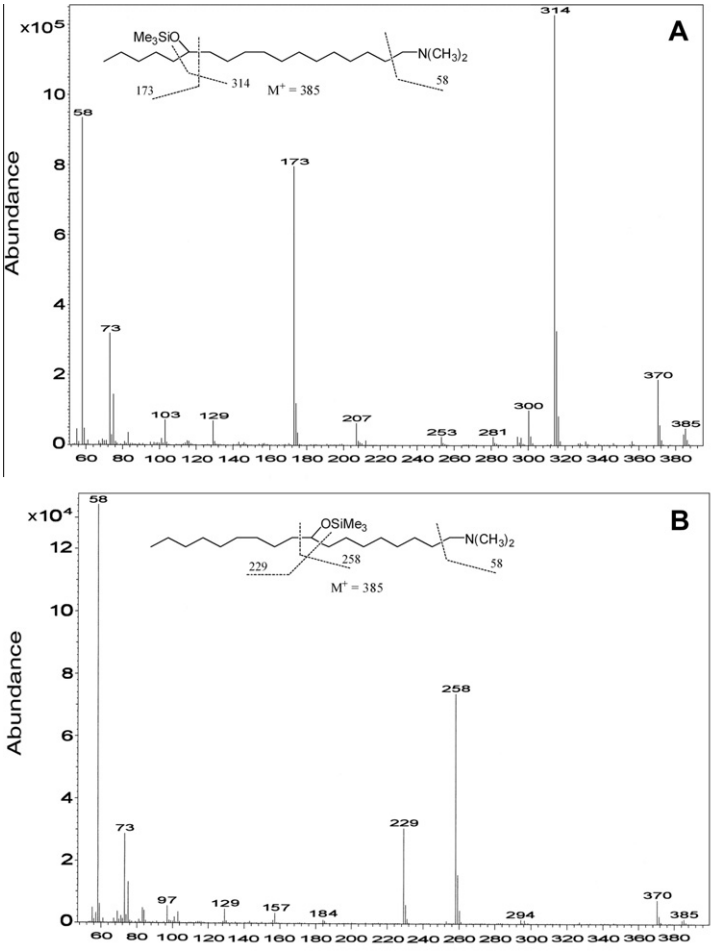


Fig. 3. Electron-impact mass spectra of derivatized products from LDMA. (A) Major product. (B) Minor product.

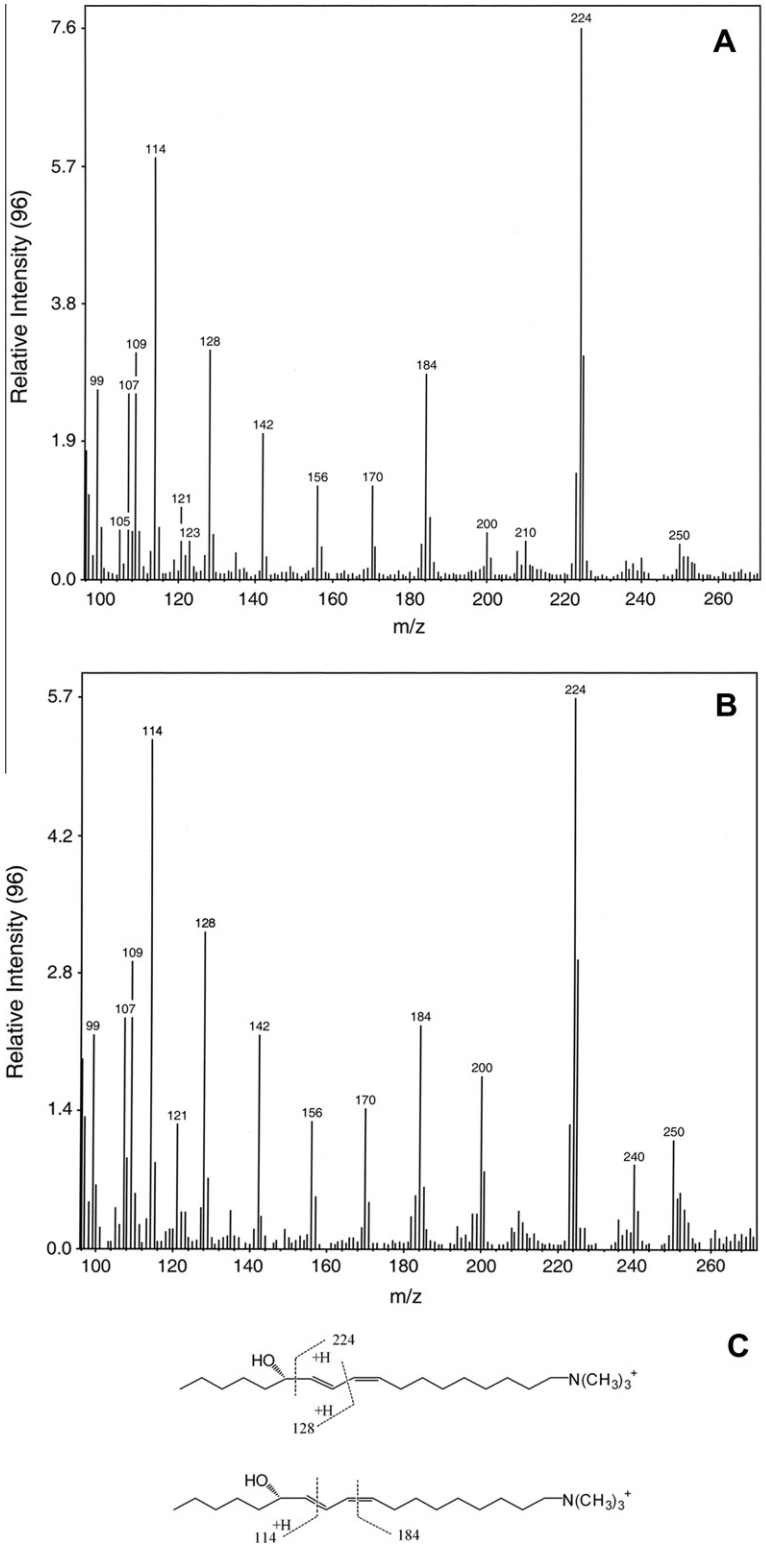


Fig. 4. Electrospray MS/MS spectra of products. (A) Reduced product from LTMA. (B) Methylated reduced product from LDMA. (C) Tentative peak assignments.

lines in the Figures. The slopes cannot be compared visually, since the scales are different). When LTMA is substrate, the slopes are identical within error in Figs. 1 and 2, which indicates that this substrate is equally active at pH 7.0 and 9.0. With LDMA as substrate, oxygenation is barely detectable at pH 9.0.

The oxygenation of LDMA catalyzed by SBLO-1 at pH 7.0 gives rise to a species with an absorption maximum at 234 nm, which is characteristic of the conjugated diene in lipoxygenase products. Assay with glutathione peroxidase [31] indicated that a peroxide had formed. To establish the structure of the product(s), the reaction was carried out on larger scale, and the products were treated with $NaBH_4$ to reduce hydroperoxides (**2a** and/or **3a**, Scheme 3) to the corresponding alcohols (**2b** and/or **3b**). The ^1H NMR spectrum of the resulting material was identical in the δ 4–7 region to the

spectrum of 13-(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoate (13-HOD), which indicates that that the product is a *cis–trans* diene with a hydroxyl group on the carbon next to the *trans* double bond. The spectrum is consistent with either **2b** or **3b**. To distinguish between these regioisomers, the product mixture was catalytically hydrogenated, trimethylsilylated and analyzed by GC/MS. The mass spectrum of the major product (Fig. 3A) was consistent with the presence of a trimethylsilyloxy group on carbon-13. Also detected was a minor product (about 3%) with a mass spectrum consistent with the trimethylsilyloxy group on carbon-9 (Fig. 3B). These results indicate that the action SBLO-1 on LDMA produces a mixture of the 13-hydroperoxide, **2a**, and the 9-hydroperoxide, **3a**, in a ratio of 97:3.

The stereochemistry of the major product was determined by a modification of procedures developed by Hamberg [34] and Gardner [35]. The $NaBH_4$-reduced product was derivatized with (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (**6**) [36], and the resulting ester was degraded as shown in Scheme 4. This procedure should convert the *S* enantiomer of **2b** to **7** and the *R* enantiomer of **2b** to **8**. GC/MS analysis of the degradation products under the conditions described in Materials and Methods gave peaks at 11.4 min and 11.2 min in a ratio of 90:10. The retention times and mass spectra were identical to those obtained on a synthetic mixture of **7** and **8** produced by reaction of (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride with racemic methyl 2-hydroxyheptanoate. The degradation shown in Scheme 4 was also carried out on (*S*)-13-HOD, and in this case, the relative intensities of the peaks at 11.4 min and 11.2 min were 91:09. This result establishes that the diasteriomer eluting at 11.4 min is **7**. Consequently, the results obtained on the products from LDMA indicate that the stereochemistry of **2a** at carbon-13 is predominantly *S*.

The oxygenation of LTMA by SBLO-1 at pH 9.0 also gave rise to a peroxide, as shown by the ferrous/xylenol orange assay [30]. Since initial studies indicated that the product could not be extracted from aqueous solution, a volatile buffer, $(NH_4)_2CO_3$, was employed for preparative experiments and removed *in vacuo*. The residue gave an NMR spectrum that contained the expected signals for either **4a** or **5a**. Following reduction by $NaBH_4$, electrospray MS gave a peak at *m/z* 325, the expected M + ion for either **4b** or **5b**. The MS/MS spectrum obtained by collision-induced fragmentation of the *m/z* 325 ion is shown in the in Fig. 4A. To facilitate the interpretation of this spectrum, the 97:3 mixture of **2b** and **3b** obtained in the experiments with LDMA was methylated with CH_3I to provide a sample that should be a 97:3 mixture of **4b** and **5b**. The electrospray MS/MS spectrum of this material (Fig. 4B) is virtually identical to the spectrum in Fig. 4A. The close correspondence of these spectra indicates that oxygenation of LTMA occurs primarily at carbon-13 to give **4a**. Tentative assignments of some of the prominent fragments in the MS/MS spectra are given in Fig. 4C. Degradation of the product from LTMA according to Scheme 4 gave **7** and **8** in a ratio of 80:20. This result indicates that the major product has predominantly the *S* configuration at carbon-13.

4. Discussion

The results with LTMA unequivocally demonstrate that SBLO-1 will oxygenate a substrate in which the carboxylate group of linoleic acid is replaced with a positively charged group. The activity is about the same at pH 9.0 and pH 7.0. The major product at pH 9.0 was identified as 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienyltrimethylammonium ion (**4a**). Thus, the reaction proceeds with the same regio- and stereochemistry as occurs with linoleate.

At pH 7.0, LDMA is oxygenated at about the same rate as LTMA, and the major product is 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienyldimethylamine (**2a**). At pH 9.0, the oxygenation of LDMA is barely detectable. Based on the reasonable assumption that the protonated form of LDMA should behave similarly to LTMA, the lower activity of LDMA at pH 9.0 is likely due to a lower concentration of protonated LDMA at pH 9.0 than at pH 7.0. Owing to the very limited solubility of LDMA in aqueous solution, its pK_a cannot be reliably determined. Smaller tertiary amines have pK_a values of about 10 [37]. As noted below, LDMA appears to aggregate under the conditions of our experiments, and this would probably perturb the ionization equilibrium in favor of the neutral species and shift the apparent pK_a to a lower value [38]. The pK_a of N-octadecylamine in neutral or cationic micelles has been found to be 8.5–9.0, compared with values of about 10.6 for shorter chain primary aliphatic amines in aqueous solution [39]. Aggregation of linoleic acid gives rise to apparent pK_a values of 7–8 [13,33], which indicate a sizeable perturbation in favor of the neutral species.

Solutions of LTMA and LDMA at micromolar concentrations at pH 7.0 and pH 9.0 are slightly opaque, indicating that these substances are not completely soluble at these concentrations. For this reason, we carried out the kinetic studies using O_2 uptake rather than spectroscopic methods. We have not examined in detail the dependence of the rates on substrate concentration, since the actual concentration of dissolved substrate is unlikely to be equal to the nominal concentration. The unprotonated form of LDMA is likely to be less soluble than the protonated form, and this may contribute to the low activity of LDMA at pH 9.0.

It is now clear that SBLO-1 will oxygenate substrates in which the polar end is either negative, positive or zwitterionic, as in the case of phospholipids. Uncharged substrates can also be oxygenated [13,40], especially if their solubility is enhanced with detergents. For example, diacylglycerides that contain linoleoyl side chains are good substrates in the presence of deoxycholate [40]. The broad specificity of SBLO-1 with respect to the polar end of its substrates is consistent with proposals that interaction of the protein with the alkyl terminus is the primary determinant of specificity [9,41].

As noted in the Introduction, the manner in which SBLO-1 binds substrates is uncertain. One possibility is that linoleate and other fatty acid substrates bind in a manner that is similar to the binding of 13-HPOD to SBLO-3, with the carboxylate of the fatty acid close to arginine-707 [19]. Docking and site-directed mutagenesis experiments provide some support for this proposal [42]. With this sort of model for substrate binding, one might expect that replacement of the negatively charged carboxylate with a positively charged group would greatly lower substrate activity owing to an unfavorable interaction with arginine-707. An alternative possibility is that the carboxylate of linoleate binds at or near the surface of the protein, and several proposals of this nature have been put forth [2,21,24,43]. Binding in this manner can explain the activity of SBLO-1 with larger substrates such as phospholipids. If the carboxylate group of linoleate binds near the protein surface, it could be stabilized either by interaction with a positively charged residue or by interaction with water. In the latter case, one would expect that replacing the carboxylate group of linoleate with a positively charged group might not result in a drastic reduction in substrate activity, since interaction with water is favorable with either a positive or negative charge. The robust activity that we observe with LTMA and LDMA is consistent with this sort of model.

At pH 7.0, the reaction catalyzed by SBLO-1 on LDMA shows a very high level of regiospecificity in favor of oxygenation at carbon-13. Only about 3% of the 9-hydroperoxide is formed. In contrast, oxygenation of linoleic acid catalyzed by SBLO-1 gives about 20% of 9-HPOD at pH 7, compared with about 5% at pH 9.0 [44]. The higher yield of 9-HPOD formed at pH 7.0 has been attributed to "reverse binding" in which neutral linoleic acid is proposed to bind with the protonated carboxyl group occupying the site

where the methyl terminus normally binds [44]. For linoleic acid, "reverse binding" is expected to become more likely as the pH is lowered and the concentration of the neutral species is increased. For LDMA, lowering the pH should reduce the concentration of the neutral species, so "reverse binding" is expected to become less favorable. The high regiospecificity of the reaction with LDMA at pH 7.0 is consistent with this prediction.

Acknowledgments

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