Controlled formation of mono- and dihydroxy-resolvins from EPA and DHA using soybean 15-lipoxygenase

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Abstract  Resolvins and protectins are important anti-inflammatory and pro-resolution compounds derived from the enzymatic oxidation of omega-3 fatty acids all‐cis‐5,8,11,14,17‐eicosapentaenoic acid (EPA) and all‐cis‐4,7,10,13,16,19‐docosahexaenoic acid (DHA). We have developed a simple, controlled method to synthesize an array of resolvin and protectin analogs from fatty acid starting materials using soybean 15‐lipoxygenase. The conditions were optimized for the production of both mono‐ and dihydroxy derivatives, with enzyme concentration and pH found to have a significant effect on the reaction products. The methods were applied to five biologically important omega‐3 and omega‐6 fatty acid substrates. Mono‐ and dihydroxy compounds were successfully synthesized from all substrates and the products were characterized by normal phase (NP) HPLC, GC‐MS, TOF‐MS, UV‐visible (UV‐vis) spectroscopy, and NMR spectroscopy.

The methods could be further applied to any polyunsaturated fatty acids containing the cis‐1,4,7,10‐undecatetraene moiety to produce a range of novel compounds with potential biological activity.—Dobson, E. P., C. J. Barrow, J. A. Kralovec, and J. L. Adcock. Controlled formation of mono‐ and dihydroxy‐resolvins from EPA and DHA using soybean 15‐lipoxygenase. J. Lipid Res. 2013. 54: 1439‐1447.

Supplementary key words  polyunsaturated fatty acids • eicosanoid • docosanoid • eicosapentaenoic acid • docosahexaenoic acid

Although generally known for their roles in energy storage and membrane structure (1), polyunsaturated fatty acids (PUFAs) such as arachidonic acid, all‐cis‐5,8,11,14‐eicosatetraenoic acid (ARA, C20:4n-6), all‐cis‐5,8,11,14,17‐eicosapentaenoic acid (EPA, C20:5n-3), and all‐cis‐4,7,10,13,16,19‐docosahexaenoic acid (DHA, C22:6n-3) are also metabolized into a range of potent lipid mediators (2, 3). These compounds are involved in regulating cellular processes including inflammation, allergies, and blood clotting. Important families of mediators metabolized from each fatty acid are summarized in Fig. 1. Most of the metabolites of omega-6 fatty acids are pro-inflammatory (the lipoxins are an important exception) and those from omega-3 fatty acids tend to be anti-inflammatory (the prostaglandins produced from EPA are pro-inflammatory, but less so than those produced from ARA) (2, 4).

Inflammation is the body’s protective response to microbial invasion, injury, and other foreign particles (5). Without inflammation, wounds and infections would never heal and progressive tissue destruction would compromise the survival of the organism (5, 6). However, uncontrolled inflammation is a major contributing factor to a number of conditions, including arthritis, Alzheimer’s disease, multiple sclerosis, cardiovascular disease, cancer, periodontal disease, and allergies (7). The noninflammatory state does not arise passively from an absence of inflammatory stimuli; rather the resolution of inflammation requires numerous biochemical signals to end the inflammatory response, including lipid mediators such as lipoxins, resolvins, protectins, and maresins (4, 6–8). Serhan and coworkers in particular have undertaken groundbreaking research in this field over the last thirty years, uncovering different families of mediators, as well as characterizing the compounds, their biological actions and

Abbreviations: ARA, arachidonic acid, all‐cis‐5,8,11,14‐eicosatetraenoic acid; DHA, all‐cis‐4,7,10,13,16,19‐docosahexaenoic acid; 7,17‐diHDPAn‐3, 7,17‐dihydroxydocosapenta‐8E,10Z,13Z,15E,19Z‐enoic acid; 10,17‐dihydroxydocosapenta‐4Z,7Z,11Z,13Z,15E,19Z‐enoic acid; 5,15‐diHPEA, 5,15‐dihydroxyeicosapenta‐6E,8Z,11Z,13E,17Z‐enoic acid; 8,15‐diHEPA, 8,15‐dihydroxyeicosapenta‐5Z,9E,11Z,13E,17Z‐enoic acid; 5,15‐diHETE, 5,15‐dihydroxyeicosatetra‐6E,8Z,11Z,13E‐enoic acid; 8,15‐diHETE, 8,15‐dihydroxyeicosatria‐5Z,9E,11Z‐enoic acid; DPAn‐3, all‐cis‐4,7,10,13,16‐docosahexaenoic acid; EPA, all‐cis‐5,8,11,14‐eicosapentaenoic acid; 17‐HDPAn‐3, 17‐hydroxydocosapenta‐7Z,10Z,13Z,15E,19Z‐enoic acid; 17‐HDPAn‐6, 17‐hydroxydocosapenta‐4Z,7Z,10Z,13Z,15E,19Z‐enoic acid; 15‐HETE, 15‐hydroxyeicosapenta‐5Z,8Z,11Z,13E,17Z‐enoic acid; 15‐HEPA, 15‐hydroxyeicosapenta‐5Z,9E,11Z,13E,17Z‐enoic acid; LOX, lipoxygenase; NP‐HPLC, normal phase HPLC.; 13‐LOX, soybean lipoxygenase; 15‐LOX, tomato lipoxygenase; 17‐LOX, P‐DHA, 17‐hydroxydocosahexa‐4Z,7Z,10Z,13Z,15E,19Z‐enoic acid; 10,17‐dihydroxydocosahexa‐4Z,7Z,10Z,13Z,15E,19Z‐enoic acid; SPE, solid phase extraction; UV‐vis, UV‐visible.

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biosynthetic pathways (9–14). These compounds are all di- and trihydroxy fatty acids with conjugated double bond systems. The omega-3 and omega-6 fatty acid precursors cannot be produced in the body and must be obtained from the diet (15).

PUFAs and the mediators synthesized from them are potentially important nutraceuticals and pharmaceutical targets for the prevention and treatment of several chronic immune diseases (16). For example, lipoxin analogs are already being developed as drugs for asthma and other inflammatory airway diseases (17), and resolvin analogs are being tested for the treatment of dry eye and other retinal diseases (Resolvys) (18). These oxygenated fatty acids are products of a number of enzyme systems; primarily lipoxygenases (LOXs), cyclooxygenases, and cytochrome P450 epoxygenases (2, 3, 19). Synthesis often involves multiple enzymes and cell types (19, 20). The products are mainly mono-, di-, and trihydroxylated products of fatty acids and the bioactivity and potency is dependent upon multiple factors including the double bond configuration, and the location and chirality of hydroxy groups (19, 21). Therefore, there is a need to synthesize these compounds with a high degree of regio- and stereospecificity. A small number of organic synthesis methods have been published [for examples see (22–25)], but these involve challenging and time-consuming multistep reactions with various stereocenters and cis/trans double bonds. The use of enzymes as biocatalysts is an attractive option to overcome the difficulties of organic synthesis for the production of a range of known and novel lipid mediators (26, 27).

Lipoxygenases are involved in the biosynthetic pathways of many lipid mediators including leukotrienes, lipoxins, resolvins, protectins, and maresins, and are therefore a valuable enzyme class to investigate as biocatalysts (26). Lipoxygenases are nonheme iron-containing dioxygenases that catalyze the regioselective and enantioselective oxidation of certain polyunsaturated fatty acids containing one or more cis,cis-1,4-pentadienoic moieties to give the corresponding hydroperoxy derivatives (26, 28–31). Lipoxygenases with different selectivities have been identified in a range of species, and many are available commercially.

If we can gain a better understanding of the reactions, we can potentially create products with higher purity and in higher yield and manipulate the reaction conditions to produce a larger range of compounds, using much simpler techniques than those required for organic synthesis. Lipoxygenase enzymes have previously been investigated for the production of hydroxy fatty acids, with most work utilizing soybean 15-lipoxygenase-1 (15-sLOX)-1 (32–35). This enzyme is probably the most widely studied and well-characterized LOX enzyme (26). It is one of at least eight lipoxygenase isoforms found in soybeans, the crystal structure is known and the enzyme is available commercially (31, 36, 37).

Single dioxygenation reactions catalyzed by lipoxygenase enzymes have mainly used linoleic acid (C18:2n-6) as substrate, with a significant amount of work also done on α-linolenic acid (C18:3n-3) and arachidonic acid, but less work has been done using other substrates (34, 35, 38–42). The first report of using a lipoxygenase enzyme to catalyze a double dioxygenation was by Bild et al. in 1977 (43, 44). They used 15-sLOX and arachidonic acid to produce 8,15-dihydroperoxy-5,9,11,13-eicosatetraenoic acid. Compared with single dioxygenation reactions, only a small number of double dioxygenation reactions have been studied, and almost all of these have used arachidonic acid or DHA as substrate (13, 32, 45–54); only one paper also utilized both all-cis-4,7,10,13,16-docosapentaenoic acid (DPAn-6) and all-cis-7,10,13,16,19-docosapentaenoic acid (DPAn-3) (33), and one paper reported the formation of a conjugated triene from EPA (55), although the structure was not determined.

In this paper we are studying double dioxygenation reactions catalyzed by 15-sLOX, initially using DHA as a model substrate for the optimization of reaction conditions. We then apply the reaction to a range of biologically important omega-3 and omega-6 PUFA substrates and characterize the products. We also compare the affinity of the enzyme for each of the substrates. This paper includes the first characterization of products produced by the double dioxygenation of EPA catalyzed by a LOX enzyme, and demonstrates the usefulness of LOXs for the production of a range of compounds structurally related to resolvins.

MATERIALS AND METHODS

Materials

Soybean 15-lipoxygenase (EC 1.13.11.33, P1, 13.7 megaunits/ml, 50 mg of protein/ml, 270 kilounits/mg of protein) and prostaglandin B2 were purchased from Cayman Chemical (Ann Arbor, MI). The fatty acids DHA, EPA, ARA, DPAn-3, and DPAn-6 were purchased from Nu-Chek Prep (Elysian, MN). Sodium tetraborate and hydrochloric acid were purchased from APS Chemicals Ltd. (Seven Hills, NSW, Australia). Sodium borohydride, Supelclean™ LC-18 solid phase extraction (SPE) tubes (5 ml, 0.5 g, Supelco Analytical), SPE vacuum apparatus (Supelco Analytical), 2,2-dimethoxypropane (98%, Fluka), platinum(IV) oxide, N,Obis (trimethylsilyl)trifluoroacetamide (with 1% trimethylchlorosilane), and other reagents were purchased from commercial sources.

Supplemental Material can be found at: http://www.jlr.org/content/suppl/2013/03/07/jlr.M036186.DC1.html
Methylated products were purified by preparative normal phase solvent extraction. Products were then loaded onto the cartridge and the cartridge was washed with deionized water and dried under vacuum. The products were then extracted by chloroform solvent extraction. Products were then methylated with 15-sLOX (5 mg added per 10 ml substrate solution) after 15 min and left with gentle stirring until foaming occurred. The reaction mixture was stopped. Products were extracted by SPE or solvent extraction. Products were then methylated with 15-sLOX (5 mg added per 10 ml substrate solution) after 15 min and left with gentle stirring until foaming stopped. Products were extracted by chloroform solvent extraction or SPE. For SPE, C18 cartridges were conditioned according to the manufacturer’s instructions. The reaction mixture was loaded onto the cartridge and the cartridge was washed with deionized water and dried under vacuum. The products were then eluted with a small quantity of ethanol. No further purification was required.

For the synthesis of dihydroxy fatty acids, the PUFA substrate (0.1 mM) was prepared in borate buffer (50 mM, pH 9) from a stock solution (10 mM in ethanol), and the reaction initiated by the addition of 15-sLOX (5 mg added per 10 ml substrate solution). The reaction then proceeded as above. Products were extracted by SPE or solvent extraction. Products were then methylated with (trimethylsilyl)diastreemethane (200 μl, 2 M in diethyl ether) and methanol (200 μl) for 1 h at room temperature. Methylated products were purified by preparative normal phase (NP) HPLC (see below).

**NP-HPLC**

An Agilent 1200 series HPLC (Agilent Technologies Australia, Mulgrave, Vic, Australia) in normal phase mode, equipped with a solvent degasser, quaternary pump, autosampler, thermostated column compartment, diode array detector, and fraction collector was used for all HPLC analyses. Analytical separations were carried out on a Supelcosil LC-Diol column (Supelco, 25 cm × 3 mm, 5 μm particle size; Sigma-Aldrich) with an isotropic mobile phase of 95% solvent A (heptane with 0.1% (v/v) acetic acid and 0.1% (v/v) 2,2-dimethoxypropane) and 5% isopropanol. The flow rate was 0.5 ml/min and the column temperature was maintained at 10°C. The sample was prepared in mobile phase and 10 μl injected. The signal was monitored at multiple wavelengths: 210 nm to detect fatty acids, 234 nm to detect conjugated dienes, and 270 nm to detect conjugated trienes.

Preparative separations were carried out on methylated products using a LiChrorpher silica column (Merck, 12.5 cm × 4 mm, 5 μm particle size) with a mobile phase gradient of 1% isopropanol to 5% isopropanol in heptane over 20 min, and held constant at 5% isopropanol for an additional 10 min. The flow rate was 1 ml/min and the column temperature was maintained at 30°C. The samples were prepared in mobile phase and 100 μl injected. Fraction collection was triggered by the signal at 250 nm. Multiple injections per sample were performed and the purified fractions pooled.

Under these conditions, fatty acid starting materials elute first, monohydroxy products elute second, and dihydroxy products elute last. These methods enabled the separation of 7,17-dihydroxy DHA and 10,17-dihydroxy DHA (both produced from the lipoxygenase catalyzed dioxygenation of DHA), which could not be achieved by reversed phase HPLC (47).

**GC-MS**

GC-MS analysis was performed on an Agilent 6890N gas chromatograph with a 5975 mass selective detector (electron impact ionization). Separations were performed on a DB-5MS column (Agilent, 30 m × 0.25 mm, 0.25 μm film thickness) with helium carrier gas (1.5 ml/min). Samples were injected (2 μl) into the inlet which was kept at 250°C with a split ratio of 20:1. The oven temperature program was as follows: 100°C for 2 min, increased to 300°C at 10°C/min, held at 300°C for 8 min. Monohydroxy- and dihydroxy-fatty acids were hydrogenated and silylated prior to injection. To hydrogenate, the sample was prepared in 2 ml methanol with 10 mg PtO2 catalyst under a stream of hydrogen with stirring for 25 min, followed by filtration (0.45 μm filter) and evaporation of the solvent under nitrogen. Compounds were then silylated in a mixture of N,O-bis(trimethylsilyl)trimethylsilyl trifluoroacetamide with 1% trimethylchlorosilane (200 μl) and pyridine (200 μl) at 100°C for 1 h.

This method did not give full resolution of the different dihydroxy isomers (other columns were tested and did not provide an improvement in separation), however mass spectra were used to determine the location of the hydroxyl groups.

**UV-visible spectrophotometry**

UV-visible absorption spectra were collected for purified products. Spectra were collected in acetonitrile using a Varian Cary 300 Bio UV-visible spectrophotometer with a 2.0 mm path length quartz cuvette. Spectra were collected from 200 nm to 300 nm in double beam mode with a bandwidth of 1 nm and scan rate of 0.2 nm/sec.

**High-resolution mass spectrometry**

The molecular masses of the purified products were determined using high-resolution mass spectrometry with an Agilent LC/MSD time-of-flight spectrometer. Direct injection of products in acetonitrile was performed with an Agilent 1200 Series HPLC binary pump and autosampler modules with a mobile phase of acetonitrile (flow rate of 1 ml/min). Mass spectrometer conditions were as follows: drying gas, nitrogen (7.1/min, 350°C); nebulizer gas, nitrogen (16 psi); capillary voltage, 4.0 kV; vaporizer temperature, 350°C; and cone voltage, 60 V. Spectra were collected using an electrospray ionization source operating in either negative or positive mode and data were processed with Agilent MassHunter Qualitative Analysis software.

**NMR experiments**

NMR experiments were performed on a Bruker Ascend 500 MHz Fourier transform (FT) NMR spectrometer or a JEOL Eclipse 400 MHz FT-NMR spectrometer. 10–50 mg of purified product was dissolved in CD3OD and spectra were collected at room temperature. The following experiments were performed: 1D 1H-NMR; 2D 1H, 1H-COSY; 1D 13C-NMR; 1D 13C-DEPT-135; 2D 13C, 13C-HMQC and 2D 13C, 13C-HMBQ. Bruker NMR data was processed using MestreNova version 7.1.0 and JEOL NMR data was processed with Delta JEOL USA processing software.

**Enzyme kinetics measurements**

The rate of formation of conjugated trienes from the different fatty acid substrates catalyzed by 15-sLOX was measured by...
monitoring the absorbance of the reaction mixture at 270 nm. To initiate the reaction, 1.5 mg of 15-sLOX (in 1 ml water) was added to a quartz cuvette containing 2 ml substrate (1 μM–0.3 mM) in oxygenated borate buffer (75 mM, pH 9). The solution was quickly mixed and absorbance measured every 0.2 min for 2 min. Reactions were performed at room temperature. Spectra were collected using a Varian Cary 300 Bio UV-visible spectrophotometer with a 10 mm path length quartz cuvette. All measurements were repeated three times. Data was fitted to the Michaelis-Menten kinetic model, \( r = \frac{V_{\text{max}} [S]}{(K_m + [S])} \) by least squares fit to give kinetic parameters (33).

RESULTS

Optimization of reaction conditions using DHA

The dioxygenation of DHA catalyzed by 15-sLOX can produce the following compounds as major products: 17S-hydro(pero)xydocosahexa-4Z,7Z,10Z,13Z,15E,19Zenoic acid (17SH(P)DHA), 10S,17Sdihydro(pero)xydocosahexa-4Z,7Z,11E,13Z,15E,19Zenoic acid (10S,17SdihP(DHA)), and 7S,17S-dihydro(pero)xydocosahexa-4Z,7Z,10Z,13Z,15E,19Zenoic acid (7S,17SdihP(DHA)) (Fig. 2) (13, 32, 33, 47). 7S,17SdihDHA is known as resolv D5 (RvD5) and has demonstrated pro-resolving activity (56), while 10S,17SdihP(DHA) (called PDX) is an isomer of (neuro)protectin D1 and also has demonstrated bioactivity (45).

Table 1. Reaction variables studied for the 15-sLOX catalyzed dioxygenation of DHA

<table>
<thead>
<tr>
<th>Variable</th>
<th>Initial Conditions</th>
<th>Range Studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme amount</td>
<td>5 mg</td>
<td>1–6 mg</td>
</tr>
<tr>
<td>Substrate concentration</td>
<td>0.10 mM</td>
<td>0.05–0.50 mM</td>
</tr>
<tr>
<td>Temperature</td>
<td>20°C</td>
<td>0–40°C</td>
</tr>
<tr>
<td>Buffer concentration</td>
<td>50 mM</td>
<td>30–70 mM</td>
</tr>
<tr>
<td>Oxygenation</td>
<td>No sparging</td>
<td>Nitrogen sparging</td>
</tr>
<tr>
<td>Time</td>
<td>15 min</td>
<td>0–60 min</td>
</tr>
</tbody>
</table>

The reaction proceeds as follows:

\[
\text{DHA} \xrightarrow{15\text{-sLOX}} \text{17S-H(P)DHA} \xrightarrow{15\text{-sLOX}} 10S,17S\text{dih(P)DHA} + 7S,17S\text{dih(P)DHA}
\]

The amount of product formed and the ratio of different products depend upon the reaction conditions. To examine this further, we monitored the product profiles under different reaction conditions (Table 1) using NP-HPLC (Fig. 3). The two dihydro(pero)xy compounds can easily be distinguished by their absorption spectra, as 10S,17S-dihP(DHA) contains a conjugated triene and has a \( \lambda_{\text{max}} \) of approximately 270 nm, while 7S,17SdihP(DHA) contains two conjugated dienes and has a split pattern with two maxima at approximately 225 and 244 nm (in heptane: isopropanol, 95:5) (47).

Enzyme and substrate concentrations. Under our conditions, at low enzyme concentrations (<5 mg/10 ml) the DHA is not fully converted into dihydro(pero)xy products, and some monohydro(pero)xy-DHA still remains (no DHA remained at the end of the reaction under any of the enzyme concentrations tested). The conversion of monohydro(pero)xy-DHA to dihydro(pero)xy products increases with increasing enzyme concentration until full conversion is achieved (5 mg/10 ml).

At low substrate concentrations (≤0.10 mM) DHA fully converts into the two dihydro(pero)xy products and all DHA is consumed. The amount of product generated is proportional to the starting amount of DHA. At DHA concentrations above 0.10 mM, the formation of dihydro(pero)xy products is completely inhibited. The DHA is still fully consumed; some is converted into 17-H(P)DHA, and two new products are also formed. The first compound (only detected for reactions using 0.50 mM DHA) eluted early in NP-HPLC (at 3.5 min), \( \lambda_{\text{max}} = 240 \) nm, and was unchanged by the sodium borohydride reduction. The second major product formed at both 0.2 mM and 0.5 mM DHA, and eluted after 17-H(P)DHA, but before the dihydro(pero)xy compounds, at 6.6 min, \( \lambda_{\text{max}} = 275 \) nm; after reduction with sodium borohydride the peak shifts to 9.5 min, \( \lambda_{\text{max}} = 235 \) nm. Adding more enzyme to compensate for the increase in DHA and maintain a constant substrate:enzyme ratio did not change the product profile. It was suspected that these products were formed due to insufficient oxygen being present in the solution to dioxygenate the higher quantities of DHA. This was confirmed by bubbling oxygen through the solution during the reaction; the additional products were no longer detected, only mono- and dihydro(pero)xy-DHA products were formed. The first eluting compound is expected to be a fatty acid dimer with a conjugated diene formed during dimerization (57), while the second compound was isolated and identified as 17-oxoheptadeca-4Z,7Z,10Z,13Z,15E-pentaenoic acid.

Reaction temperature. At low temperatures (<20°C), the monohydro(pero)xy-DHA was not fully converted into dihydro(pero)xy products. Between 10°C and 30°C, there was not a significant difference in the amount of
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Concentration did not have a significant effect on the reaction over the range studied.

Oxygen concentration. Saturing the buffer with oxygen before the addition of enzyme did not have any effect on the product profile at this substrate concentration. However, it does have an effect under conditions where oxygen is the limiting reagent, including high substrate concentrations. Nitrogenating the buffer before the reaction and performing the reaction in a sealed container to limit oxygen from the reaction, completely stopped the formation of dihydro(pero)xy compounds and significantly reduced the formation of 17-H(P)DHA. Instead, the main product formed was the same compound seen in reactions with high substrate concentrations, 17-oxoheptadeca-4Z,7Z,10Z,13Z,15E-pentaenoic acid. Small amounts of other unidentified products were also detected.

Reaction time. Under the initial conditions shown in Table 1, the reaction was performed and aliquots taken at regular intervals to monitor the time course of the reaction. It was found that all DHA was converted into 17-H(P)DHA immediately (within 10 s) after the addition of the enzyme. The 17-H(P)DHA was quickly converted into dihydro(pero)xy products and the reaction was complete within 5 min.

Buffer pH and concentration. Reaction pH is known to have a significant effect on lipoxygenase reactions (26, 28, 34, 42–44, 53–55, 58) and this was also found for the double dioxygenation reactions studied in this paper (Fig. 4). Total conversion of 17-H(P)DHA to dihydro(pero)xy compounds was observed at pH 9 and below. The total amount of dihydro(pero)xy compounds produced increased to a maximum at pH 9, however, all the initial substrate is not accounted for at low pH. That is, there is less product, but no starting material remains. The fate of the missing substrate at low pH is currently unknown, as no new products were detected by NP-HPLC.

From pH 9 to 12, the amount of dihydro(pero)xy compounds produced decreased, until there was none produced at pH 12 and above. Over this same range the amount of monohydro(pero)xy-DHA increases from undetectable at pH 9 to a maximum at pH 12.5. At pH 13 the amount of 17-H(P)DHA produced decreased, and significant amounts of unreacted DHA remained. There were also small amounts of two unidentified compounds produced at pH 11 and above, most likely containing a conjugated diene, and of similar polarity to the dihydro(pero)xy products. The ratio of 10,17-diH(P)DHA to 7,17-diH(P)DHA increases up to pH 10 then drops severely. The buffer concentration did not have a significant effect on the reaction over the range studied.

Oxygen concentration. Saturing the buffer with oxygen before the addition of enzyme did not have any effect on the product profile at this substrate concentration. However, it does have an effect under conditions where oxygen is the limiting reagent, including high substrate concentrations. Nitrogenating the buffer before the reaction and performing the reaction in a sealed container to limit oxygen from the reaction, completely stopped the formation of dihydro(pero)xy compounds and significantly reduced the formation of 17-H(P)DHA. Instead, the main product formed was the same compound seen in reactions with high substrate concentrations, 17-oxoheptadeca-4Z,7Z,10Z,13Z,15E-pentaenoic acid. Small amounts of other unidentified products were also detected.

Reaction with a range of substrates

From the above set of experiments, two sets of optimized reaction conditions were chosen: one for the production of monohydro(pero)xy products and one for the production of dihydro(pero)xy products (summarized in Table 2). The optimized reaction conditions were applied to a number of polyunsaturated fatty acid substrates (ARA, EPA, DPAn-6, DPAn-3, and DHA) and all compounds were found to be suitable substrates for the lipoxygenase enzyme. The reaction products are summarized in Table 3. It is interesting to note that the reaction with both docosapentaenoic acids (DPAn-6 and DPAn-3) resulted in the formation of different product ratios compared with the other substrates. While the reactions with ARA, EPA, and DHA resulted in approximately equal amounts of both dihydro(pero)xy products, the reaction of DPAn-3 produced...
only 7,17-dihydroxydocosapenta-8E,10Z,13Z,15E,19Zenoic acid (7,17-diHDPAn-3), and the reaction of DPAn-6 produced significantly more 10,17-dihydroxydocosapenta-4Z,7Z,11E,13Z,15E-enoic acid (10,17-diHDPAn-6) than 7,17-diHDPAn-6 (approximately 3:1). All compounds were characterized by NP-HPLC, GC-MS, TOF-MS, UV-visible (UV-vis) spectrometry (Table 4), and NMR (supplementary Tables I–VI).

NMR
Protons were assigned using integrated 1D 1H-NMR and 2D 1H, 1H-COSY spectra to detect correlation between neighboring protons. The classification of primary (CH3), secondary (CH2), and tertiary (CH) carbons was determined using the 13C-DEPT-135 experiments. Protons were then assigned to carbons using 2D 1H, 13C-HMQC spectra showing single through-bond relationships, and 2D 1H, 13C-HMBC spectra to identify relationships through either two or three bonds. NMR data is provided in the supplementary material (Tables I–VI).

The predicted E,Z,E double bond structure of the conjugated triene of 8,15-dihydroxyeicosatetra-5Z,9E,11Z,13E-enoic acid (8,15-diHETE), 8,15-dihydroxyeicosapenta-5Z,9E,11Z,13E,17Zenoic acid (8,15-diHEPA), 10,17-diHDPAn-6, and 10,17-diHDHA was confirmed by several characteristic proton and carbon resonances in 1D 1H-NMR, 1D 13C-NMR, and 2D 1H, 1H-COSY spectra (32, 33). First a proton resonance with a chemical shift (δ) of 6.12–6.24 ppm was absent in the 1D 1H-NMR spectrum. This chemical shift is indicative of a conjugated E,E double bond fragment, therefore absence confirms E,Z geometry. Second, the 2D 1H, 1H COSY experiments showed a correlation between protons of both CH–OH (1H δ ~4.1 ppm) carbons and protons of the adjacent –CH= with δ ~5.7 ppm, a characteristic chemical shift of trans protons. No correlation was observed between the CH–OH protons with protons at δ 5.9–6.0 ppm, indicative of the cis double bond protons located at the second position of the triene. The double bonds adjacent to the hydroxylated carbons were both identified as trans,cis, and the absolute stereochemistry was identified as E,Z,E.

The structure of the conjugated diene of all five monoxygen products, 15-hydroxyeicosatetra-5Z,8Z,11Z,13E-enoic acid (15-HETE), 15-hydroxyeicosapenta-5Z,8Z,11Z,13E,17Z-enoic acid (15-HEPA), 17-DPAn-6, 17-DPAn-3, and 17-HDHA, were confirmed as cis,trans based on the coupling constants of the double bond protons (32, 33). In 15-HETE and 15-HEPA, the value of J1,12 was ~11 Hz, indicating the presence of a cis configuration double bond. Whereas the coupling constant of J13,14 was approximately 15.2 Hz, characteristic of trans geometry. Likewise in 17-HDHA, 17-hydroxyeicosapenta-7Z,10Z,13Z,15E,19Zenoic acid (17-HDPAn-3), and 17-hydroxyeicosapenta-4Z,7Z,10Z,13Z,15E-enoic acid (17-HDPAn-6), J15,16 was approximately 11 Hz and J13,14 was 15.0–15.2 Hz.

**Reaction kinetics**

The reaction kinetics for the formation of conjugated trienes was monitored by UV-vis spectroscopy at 270 nm. The results are shown in Table 5 (DPAn-3 was not included because it did not produce significant amounts of the conjugated triene product). Because of the strong overlap of the UV absorption spectra of the monohydroxy products and the conjugated diene dihydroxy products, direct monitoring of the formation of these products was not possible (47).

From the kinetics results, DHA and DPAn-6 appear to be the best substrates for the formation of a dihydro(peroxy)xy conjugated triene product. The reaction proceeded fastest with these substrates (Vmax and km), with better efficiency and specificity (kcat/km), and with higher substrate capture (Vmax/km). However, the low km value for AA suggests the enzyme has a higher affinity for this substrate.

**DISCUSSION**

The dioxygenation of polyunsaturated fatty acids containing all-cis-1,4,7-octatriene and all-cis-1,4,7,10-undecatetraene moieties catalyzed by 15-sLOX has been shown to produce double dioxygenation products as well as the expected single dioxygenation products (13, 32, 33, 43–55). It has been proposed that the reaction proceeds through an initial, very fast, and specific dioxygenation reaction to produce the omega-6 hydroperoxy product, where the

**Table 2. Optimized reaction conditions for the production of monohydro(pero)xy and dihydro(pero)xy products**

<table>
<thead>
<tr>
<th>Enzyme amount</th>
<th>Monohydro(pero)xy</th>
<th>Dihydro(pero)xy</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mg</td>
<td>5 mg</td>
<td></td>
</tr>
<tr>
<td>Substrate concentration</td>
<td>0.10 mM</td>
<td>0.10 mM</td>
</tr>
<tr>
<td>Temperature</td>
<td>20°C</td>
<td>20°C</td>
</tr>
<tr>
<td>pH</td>
<td>pH 12</td>
<td>pH 9</td>
</tr>
<tr>
<td>Buffer concentration</td>
<td>50 mM</td>
<td>50 mM</td>
</tr>
<tr>
<td>Oxygenation</td>
<td>No oxygenation</td>
<td>No oxygenation</td>
</tr>
<tr>
<td>Time</td>
<td>15 min</td>
<td>15 min</td>
</tr>
</tbody>
</table>

**Table 3. Major products from the lipoygenase catalyzed dioxygenation of a range of polyunsaturated fatty acids**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Monohydroxy Product</th>
<th>Dihydroxy Product (conjugated diene)</th>
<th>Dihydroxy Product (conjugated triene)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARA, 20:4n-6</td>
<td>15-HETE</td>
<td>5,15-diHETE</td>
<td>8,15-diHETE</td>
</tr>
<tr>
<td>EPA, 20:5n-3</td>
<td>15-HEPA</td>
<td>5,15-diHEPA</td>
<td>8,15-diHEPA</td>
</tr>
<tr>
<td>DPAn-6, 22:5n-6</td>
<td>17-DPAn-6</td>
<td>10,17-diDPAn-6</td>
<td></td>
</tr>
<tr>
<td>DPAn-3, 22:5n-3</td>
<td>17-DPAn-3</td>
<td>17-DPAn-3</td>
<td></td>
</tr>
<tr>
<td>DHA, 22:6n-3</td>
<td>17-HDHA</td>
<td>7,17-diHDHA</td>
<td>10,17-diHDHA</td>
</tr>
</tbody>
</table>

a7,17-diDPAn-6 was detected as a minor product only.

b10,17-diDPAn-3 not detected.
(anionic) substrate enters the enzyme methyl group first. The second dioxygenation is slower and less specific, adding a hydroperoxy group at either the omega-13 or omega-16 position to form a dihydro(pero)xy product. In this instance, it is proposed that the (neutral) substrate enters the enzyme carboxylic acid group first (42, 52). This is supported by the current results examining the optimum pH of the reaction, where the second dioxygenation was shown to be suppressed at high pH values (55), as the fatty acid would be fully deprotonated (pK\text{a} of DHA under reaction conditions is \sim 7.4), and the anionic form of the substrate is unable to enter the enzyme acid group first.

Consistent with previous results, higher enzyme concentrations are required to catalyze the formation of dihydro(pero)xy products than are required to perform a single dioxygenation (32, 43, 48). This may be because at lower enzyme concentrations, enzyme deactivation/inhibition occurs before there is a chance to perform the second dioxygenation, or because the much slower kinetics of the reaction requires more enzyme to increase the probability of the reaction occurring.

At high substrate concentrations, the formation of dihydro(pero)xy compounds is completely inhibited and the formation of monohydro(pero)xy compounds is significantly reduced. This could not be rectified by the addition of more enzyme. Therefore, it is most likely that at high substrate concentrations, oxygen becomes the limiting reagent and prevents the reaction from occurring (42, 59). Instead, other products are formed, including one compound identified as 17-oxoheptadeca-4Z,7Z,10Z,13Z,15E-pentaenoic acid, and a second compound proposed to be a DHA dimer (with conjugated diene). It is most likely that these products are formed through the anaerobic lipoxygenase pathway, where a substrate free radical can be released from the enzyme without oxygenation. This can occur, because hydrogen abstraction occurs before molecular oxygen enters the reaction (60). This free radical can then react with other fatty acid molecules, radicals, and oxygen. Products such as aldehydes, ketones, and fatty acid dimers have been reported to form under anaerobic conditions (26, 57). The formation of these products could be prevented by bubbling oxygen through the solution during the reaction when high substrate concentrations were used. It has been reported previously, that oxygen pressure can allow the use of higher substrate concentrations (34). Similar products, especially 17-oxoheptadeca-4Z,7Z,10Z,13Z,15E-pentaenoic acid, were also observed in reactions where oxygen had been removed by sparging the buffer with nitrogen prior to adding the enzyme. At low oxygen concentrations small amounts of nonspecific mono- and dihydro(pero)xy products also formed (34, 60).

Although it has previously been reported that lipoxygenase catalyzed reactions should be carried out at low temperatures to prevent the formation of nonspecific oxidation and isomerization products (due to nonenzymatic reactions) (32, 34), we observed that at higher temperatures the reaction proceeded faster without an increase in unwanted side reactions. However, at 40°C, there was a decrease in the amount of product formed, either due to deactivation of the enzyme or degradation of the product.

pH was found to be a critical variable in the lipoxygenase catalyzed reactions studied. The double dioxygenation reaction can be completely suppressed by performing the reaction at pH 12, which is much higher than the optimum

### Table 4. Product characterization

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>GC-MS, Major Ions</th>
<th>Expected (m/z)</th>
<th>Actual (m/z)</th>
<th>UV-vis (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARA</td>
<td>15-HETE</td>
<td>73, 117, 173, 401, 457</td>
<td>357.24092'</td>
<td>357.22519</td>
<td>236.6</td>
</tr>
<tr>
<td></td>
<td>5,15-diHETE</td>
<td>73, 173, 261, 399, 489, 545</td>
<td>373.23493'</td>
<td>373.22115</td>
<td>243.4 (shoulder at 226.9)</td>
</tr>
<tr>
<td></td>
<td>8,15-diHETE</td>
<td>73, 173, 303, 399, 489, 545</td>
<td>373.23495</td>
<td>373.23905</td>
<td>269.0 (suppressed maxima at 259.6 and 279.5)</td>
</tr>
<tr>
<td>EPA</td>
<td>15-HEPA</td>
<td>73, 173, 173, 401, 457</td>
<td>317.21222</td>
<td>317.57493</td>
<td>235.4</td>
</tr>
<tr>
<td></td>
<td>5,15-diHEPA</td>
<td>73, 173, 261, 399, 489, 545</td>
<td>371.21928'</td>
<td>371.20427</td>
<td>244.3 (shoulder at 225.8)</td>
</tr>
<tr>
<td></td>
<td>8,15-diHEPA</td>
<td>73, 173, 303, 399, 489, 545</td>
<td>371.21928'</td>
<td>371.20417</td>
<td>269.5 (suppressed maxima at 260.4 and 280.3)</td>
</tr>
<tr>
<td>DPan-6</td>
<td>17-HDPAn-6</td>
<td>73, 117, 173, 429, 489</td>
<td>345.24297'</td>
<td>345.37750</td>
<td>228.2</td>
</tr>
<tr>
<td>DPan-3</td>
<td>17-HDPAn-3</td>
<td>73, 117, 173, 331, 429, 472, 517, 573</td>
<td>390.25113</td>
<td>399.48680</td>
<td>269.0 (suppressed maxima at 259.8 and 280.0)</td>
</tr>
<tr>
<td>DHA</td>
<td>17-HDHA</td>
<td>73, 117, 173, 429, 489</td>
<td>345.24297'</td>
<td>345.37657</td>
<td>226.5</td>
</tr>
<tr>
<td></td>
<td>7,17-diHDPAn-3</td>
<td>73, 117, 173, 289, 402, 517, 573</td>
<td>399.25058</td>
<td>399.24808</td>
<td>243.6 (shoulder at 227.6)</td>
</tr>
<tr>
<td></td>
<td>10,17-diHDPAn-6</td>
<td>73, 117, 173, 429, 472, 517, 573</td>
<td>397.23495</td>
<td>397.21042</td>
<td>243.8 (shoulder at 227.6)</td>
</tr>
</tbody>
</table>

### Table 5. Reaction kinetics for the 15-LOX catalyzed double dioxygenation of a range of polyunsaturated fatty acid substrates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ARA</th>
<th>EPA</th>
<th>DPA n-6</th>
<th>DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>V\text{max} (\mu M/min)</td>
<td>4.2</td>
<td>7.0</td>
<td>34.9</td>
<td>26.6</td>
</tr>
<tr>
<td>K\text{m} (\mu M)</td>
<td>18.8</td>
<td>53.3</td>
<td>62.4</td>
<td>49.5</td>
</tr>
<tr>
<td>V\text{max}/K\text{m} (min^{-1})</td>
<td>0.22</td>
<td>0.13</td>
<td>0.56</td>
<td>0.54</td>
</tr>
<tr>
<td>k\text{cat} (min^{-1})</td>
<td>0.8</td>
<td>1.3</td>
<td>6.5</td>
<td>4.9</td>
</tr>
<tr>
<td>k\text{cat}/K\text{m} (\mu M^{-1}/min^{-1})</td>
<td>0.94</td>
<td>0.92</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>
for the enzyme [pH 9 (43, 44, 58)], but for the purposes of synthesizing standards, ensures a more pure product. The optimum pH found for the double dioxygenation reaction (pH 9) is higher than the pH optimum reported previously for the second dioxygenation when using the mono-hydroperoxy compound as the starting material instead of the fatty acid [pH 7.5 (44, 58)]. This was thought to be due to the need to first form 17SHPDHA when using DHA as a starting material, which occurs at higher pH due to the requirement of a charged substrate, while the second dioxygenation requires a neutral substrate. However, under our conditions, we found the optimum pH for the transformation of 17SHPDHA to dihydro(pero)xy products was also pH 9, although the maximum was not as distinct and similar amounts of dihydro(pero)xy products were formed at pH 7 and 8 (results not shown). Higher pHs also improve the solubility of the substrate, which may be required under these conditions where no surfactant is used.

Under our conditions, with relatively high enzyme concentrations (compared with previous work), the initial dioxygenation reaction to form 17SHPDHA happened almost instantaneously. The second dioxygenation was also fast, consuming all 17SHPDHA within 5 min, after which the product profile remained constant.

Under certain reaction conditions, such as at high temperatures and low pH, there was a decrease in the amount of dihydro(pero)xy products produced, without a corresponding increase in another compound as detected by NP-HPLC. At this point the reason for this is unknown, but we propose that it could be caused by the formation of volatile breakdown products that are lost before analysis or are not detected by NP-HPLC, or the formation of polar compounds that are removed by SPE prior to analysis. We are continuing to investigate this.

As was expected, and as has been observed previously, the lipoxigenase catalyzed dioxygenation of other similar polyunsaturated fatty acids (AA, EPA, and DPAn-6) produced two dihydro(pero)xy products, equivalent to those produced from DHA. When DPAn-3 was utilized as a substrate however, a change in specificity of the second dioxygenation was observed, with only 7,17-diHDPAn-3 being formed in a significant quantity. Similarly, while the reaction with DPAn-6 did produce two dihydro(pero)xy products, 10,17-diHDPAn-6 was produced in excess compared with 7,17-diHDPAn-6 (estimated to be 3:1), whereas when DHA, AA, and EPA were utilized as substrates the product ratio was estimated to be 1:1. The reason for the change in specificity for the second dioxygenation when employing DPA (omega-3 or omega-6) as substrate is currently unknown. The kinetics data suggest that DHA and DPAn-6 are the best substrates for the formation of a dihydro(pero)xy conjugated triene product, but the enzyme may have a higher affinity for AA and DPAn-3.

**CONCLUSIONS**

The lipoxigenase enzyme is a valuable biocatalyst for the synthesis of a range of hydroxy fatty acid species in a regio- and stereospecific manner. We have demonstrated the use of this enzyme in a simple and controlled method for the synthesis of both mono- and dihydroxy resolvins analogs from a range of biologically important polyunsaturated fatty acids. Compounds of this class are important, as many have been demonstrated to have significant bioactivity, especially in relation to modulating the inflammatory response. The reaction can be controlled by modifying reaction conditions; dihydro(pero)xy products can be formed by using high enzyme concentrations and dihydro (pero)xy product formation can be completely inhibited by using high pH to form highly pure mono(hydro(pero)xy products instead. It is very important to maintain adequate oxygen levels throughout the reaction to prevent the formation of anaerobic reaction products.

**REFERENCES**


