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New Biotechnology, Volume 41, 2018, pages 25-33

The final published version of this manuscript can be found at:
https://doi.org/10.1016/j.nbt.2017.11.005

Soy Flour as an Alternative to Purified Lipoxygenase for the Enzymatic Synthesis of Resolvin Analogues

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ABSTRACT

Specialized pro-resolving mediators are lipid signaling molecules synthesized from omega-3 and -6 polyunsaturated fatty acids, which promote the resolution of the inflammatory response. They are potential drug targets for the treatment of numerous conditions linked with uncontrolled inflammation. Many of these mediators can be effectively synthesized using enzymes, such as lipoxygenases. However, these enzymes are expensive to purchase and can be difficult to isolate. In this work, we show that commercial soy flour can be used directly as a source of lipoxygenase for the biosynthesis of specialized pro-resolving mediators from DHA and other biologically important fatty acids. The reaction was optimized and the products characterized. We found that the reaction yield and products were comparable to those synthesized using a commercial 15-lipoxygenase preparation.

Keywords: Lipid mediator, Polyunsaturated fatty acid, Omega-3 fatty acid, Lipoxygenase, Specialized pro-resolving mediator, Oxylipin

Abbreviations:

17*S*-hydroxydocosahexa-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-enoic acid (17*S*-HDHA); 7*S*,17*S*-dihydroxydocosahexa-4*Z*,8*E*,10*Z*,13*Z*, 15*E*,19*Z*-enoic acid (7*S*,17*S*-diHDHA; RvD5); 10*S*,17*S*-dihydroxydocosahexa- 4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid (10*S*,17*S*-diHDHA; PDX); *all cis*-docosa-4,7,10,13,16,19-hexaenoic acid (DHA); *all cis*-eicosa-5,8,11,14,17-pentaenoic acid (EPA); lipoxygenase (LOX); specialized pro-resolving mediator (SPM)

INTRODUCTION

Oxylipins, including the eicosanoids in animals and the jasmonates in plants, are important biochemicals and many have physiological significance [1-3]. Oxylipins are biosynthesized by the oxidation of fatty acids through both enzymatic and non-enzymatic pathways [4-6], and include hydroperoxy-, hydroxy-, oxo- and epoxy-fatty acids.

Lipoxygenases (LOX) are key enzymes in the synthesis of hydroperoxy-fatty acids in both plants and animals [7-9]. They catalyze the dioxygenation of polyunsaturated fatty acids containing one or more *cis*,*cis*-1,4-pentadienoic moieties to form lipid hydroperoxides. The reactions have high positional and chiral specificity, with the specific substrate and product dependent upon the LOX isoform.

Hydroperoxy-conjugated fatty acids, such as those produced by lipoxygenase enzymes, are versatile chemical intermediates [10,11]. They can be reduced, dehydrated, hydrogenated, cyclized and dimerized to yield products with potential applications in coatings, plasticizers, lubricants and emulsifiers [10]. They can also be used for the synthesis of prostaglandins, resolvins and other eicosanoids and docosanoids [12]. Therefore, there is significant commercial interest in these compounds for use in pharmaceuticals, nutraceuticals, flavors and fragrances, chemical precursors, and other industrial applications.

Soy flour has been used directly as a source of lipoxygenase in a small number of studies (see Table 1), for reaction with linoleic and linolenic acids as well as oils composed predominantly of C18 fatty acids, (e.g. safflower oil, soybean oil and corn oil), for the production of mono-hydro(pero)xy fatty acids and related flavor and fragrance compounds. Both free and esterified fatty acids have been used as substrates, and both full fat and de-fatted soy flour [13] have been found to be effective LOX sources.

However, the use of soy flour for the conversion of biologically important long chain polyunsaturated fatty acids (LC-PUFAs), such as *all cis*-docosa-4,7,10,13,16,19-hexaenoic acid (DHA) and *all cis*-eicosa-5,8,11,14,17-pentaenoic acid (EPA) into specialized pro-resolving mediators (SPMs) and

related analogues has not been investigated. These compounds, including resolvins, protectins, maresins and lipoxins, are of interest because of their role in the inflammatory response [27]. Inflammation is part of the body's natural defense system, but uncontrolled and chronic inflammation are causal factors in numerous diseases and conditions, such as arthritis, Alzheimer's disease, cardiovascular disease and allergies [27,28]. Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen and naproxen, are commonly used to treat inflammation and pain [29]. However, use of these compounds is associated with a number of side-effects including gastrointestinal toxicity and cardiovascular toxicity [29].

The naturally occurring SPMs and their analogues actively trigger resolution of the inflammatory response, protecting tissues and restoring homeostasis, and may be an effective alternative treatment for inflammatory conditions [2]. However the synthesis of these compounds can be demanding due to the presence of multiple double bonds and chiral centers. Typical synthetic pathways for these compounds can range from 10 to more than 20 steps [30-32]. As an alternative, enzymes can be used to catalyze their synthesis from fatty acid substrates, with high product specificity in relation to chirality and geometry. However, there are only a small number of enzymes commercially available and they can be prohibitively expensive for use in synthesis. Soy flour is a potentially useful alternative to purified enzymes, as it is inexpensive, readily available and has high enzyme activity. Improved synthetic methods would facilitate further research into mechanisms and function of these compounds.

In this paper we demonstrate the use of soy flour as a source of lipoxygenase for the synthesis of SPMs and related analogues from LC-PUFAs. We show that soy flour is as effective as a commercial lipoxygenase preparation for catalyzing this transformation, giving similar yield and purity. This is the first time soy flour has been used as a lipoxygenase source for the dioxygenation of 20 and 22 carbon fatty acid substrates and the first time it has been used for the synthesis of dihydroperoxy fatty acids. The reaction was optimized and the products characterized. The method is suitable for the production of these compounds for pharmaceutical and nutraceutical purposes at much lower cost than using purified enzymes or total chemical synthesis methods.

MATERIALS AND METHODS

Materials

Soy flour (Kialla Pure Foods) was purchased from Organic Wholefoods (Brunswick East, VIC, Australia). Soybean 15-lipoxygenase (15-sLOX-1; EC 1.13.11.33, P_1, 9.42 megaunits/mL, 15.2 mg of protein/mL, 0.62 megaunits/mg of protein; Cayman Chemical), 7*S*,17*S*-dihydroxy DHA (Cayman Chemical), 10*S*,17*S*-dihydroxy DHA (Cayman Chemical) and prostaglandin B2 (PGB2; Cayman Chemical) were purchased from Sapphire Bioscience (Redfern, NSW, Australia). Fatty acids (Nu-Chek Prep) were purchased from Adelab Scientific (Thebarton, SA, Australia). Dess-Martin periodinane (DMP) was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

Colorimetric test for the detection of LOX isozymes

Three visual screening tests developed by Suda et al. [33] were used to detect individual lipoxygenase isozymes in the soy flour and commercial enzyme preparations. Briefly, the three tests were as follows: Test I for LOX-1: Sodium linoleate and methylene blue in tetraborate buffer (pH 9). Color change recorded within 3 min. Test II for LOX-2: Sodium linoleate, dithiothreitol, methylene blue and acetone in phosphate buffer (pH 6). Color change recorded within 5 min. Test III for LOX-3: Sodium linoleate, β-carotene and acetone in phosphate buffer (pH 6.6). Color change recorded within 3 min.

Soy flour suspension was prepared as 0.1 g (test I and III) or 0.2 g (test II) in 20 mL water. The commercial 15-sLOX-1 enzyme was diluted in water; 140 μg (test I and III) or 280 μg (test II) in 0.5 mL. For each test 0.5 mL of the enzyme solution (commercial enzyme or soy flour) and 2 mL of the dye-substrate solution were used.

Lipoxygenase catalyzed reactions

Conditions used for the lipoxygenase catalyzed reactions were based on the methods of Luquet et al. [15] and Dobson et al. [34]. Stock solutions of the fatty acid substrates (10 mM) were prepared in ethanol and diluted into the aqueous buffer as required. Unless otherwise stated, a reaction volume of

10 mL was used. The soy flour was prepared by suspending 1 g of soy flour in 20 mL water, shaking for one minute and allowing the soy flour to settle for at least 3 min. Aliquots of the solution were used for the reactions without disturbing the settled soy flour. The solution should be used between 20 min to two hours after preparation for optimum activity. Reactions were initiated by the addition of the soy flour solution and were stirred for the duration of the reaction. Where surfactants were used, they were added prior to the addition of the soy flour solution. To alter the dissolved oxygen concentration the buffer was sparged with nitrogen, air or oxygen gas for 5 min as required. Oxygen concentration of the buffer solutions was measured using a dissolved oxygen meter (Mettler Toledo, Port Melbourne, VIC, Australia). To adjust reaction temperature, the reactions were performed in ice or water baths as required.

The reactions were stopped by the addition of sodium borohydride (1 M solution in water, 200 μL for a 10 mL reaction), which reduced the hydroperoxides to hydroxy groups. This was stirred for 15 min, the mixture then acidified with glacial acetic acid (50 μL for a 10 mL reaction) and stirred for an additional 30 min. Products were extracted by solid phase extraction (SPE) under vacuum (Vac Elut SPE vacuum apparatus, Agilent Technologies, Mulgrave, VIC, Australia) or by solvent extraction. Prostaglandin B2 was added as an internal standard (20 μL of 1 mg/mL solution in ethanol) prior to extraction as required.

For SPE, C18 cartridges (Agilent Bond Elut C18 INT SPE cartridges, 500 mg, 3 mL; Agilent Technologies) were conditioned with 1 column volume of methanol and 1 column volume of water. The sample was loaded onto the column and washed with 1 column volume of water. The column was dried under vacuum for 5 to 15 min and the product eluted with 1 column volume of ethanol. The solvent was evaporated under vacuum or a stream of nitrogen.

For reactions using higher volumes of the soy flour solution (≥300 μL), solvent extraction was used instead of SPE as the soy flour blocked the SPE columns. The reaction mixture was adjusted to pH 3 with hydrochloric acid (4 M) and extracted with diethyl ether (2 × 10 mL). The diethyl ether fractions were combined and the solvent evaporated under vacuum.

Commercial 15-sLOX-1 reactions with DHA were performed as described by Dobson et al. [34]. Products were extracted by SPE as described above.

NP-HPLC

Products of the lipoxygenase catalyzed reactions were separated by normal phase HPLC [34] using an Agilent Technologies 1200 series instrument (Agilent Technologies), including solvent degasser, quaternary pump, autosampler, thermostated column compartment and diode array detector (DAD). An LC-diol column (25 cm × 3 mm, 5 µm particle size; Supelco Analytical, Sigma-Aldrich, Castle Hill, NSW, Australia) was used. The mobile phase composition was 95% v/v solvent A (heptane with 0.1% v/v acetic acid, 0.1% v/v 2,2-dimethoxypropane) and 5% v/v solvent B (isopropanol). Samples were separated under isocratic conditions within 35 min at 10ºC with a flow rate of 0.5 mL/min. Elution of mono- and di-hydroxy products was monitored spectrophotometrically at 210 nm, 234 nm, 250 nm, 270 nm and 280 nm. Samples were prepared in mobile phase and the injection volume was 15 µL. Reproducibility was confirmed by repeat injections. Data was processed using Agilent Technologies ChemStation for LC 3D system software (B.04.01).

GC-MS (including derivatisation)

Positions of hydroxy groups on the reaction products were determined by GC-MS after hydrogenation and conversion to trimethylsilylated derivatives (see below). An Agilent Technologies 6890N Network GC system with 5975 mass selective detector (electron impact) and BPX5 column (30 m × 0.25 mm, 0.25 µm film thickness; SGE, Ringwood, VIC, Australia) was used. Injection volume was 2 µl, helium carrier gas, flow rate 1.2 ml/min (average velocity of 42 cm/second) with an inlet temperature of 250ºC and a split ratio of 20:1. Initial oven temperature was 150ºC held for 2 min, increased to 300ºC at a rate of 5ºC/min over 30 min and held for 13 min. Total ion chromatograms were collected and data processed with Agilent Technologies MSD ChemStation (D.02.00.275).

Hydrogenation

Reaction products were extracted into diethyl ether by solvent extraction. The solvent was evaporated under nitrogen gas and the product re-dissolved in 2 mL ethanol. PtO_2 catalyst (10 mg) was added and

the reaction took place with stirring under a stream of hydrogen for 25 min. The mixture was filtered through a 0.45 µm filter and solvent evaporated under nitrogen.

Silylation

After hydrogenation, 200 µl of pyridine and 200 µl of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; with 1% trimethylchlorosilane) was added and the mixture heated at 100ºC for 1 hour. The samples were then analyzed by GC-MS.

Chiral RP-HPLC

Chirality of the reaction products was determined by chiral RP-HPLC after methylation (see below). An Agilent Technologies 1260 Infinity series instrument, including solvent degasser, quaternary pump, autosampler, thermostated column compartment and diode array detector (DAD) with a Lux 5µm Amylose-2 column (100 × 4.6 mm, 5 µm particle size; Phenomenex, Lane Cove, NSW, Australia) was used. Mobile phase consisted of solvent A (acetonitrile) and solvent B (0.1% v/v trifluoroacetic acid). The four 7,17-diHDHA methyl ester stereoisomers were separated using a solvent gradient of 40% to 47.5% solvent A over 45 min at 1 mL/min, 40ºC. The four 10,17-diHDHA methyl ester stereoisomers were separated under gradient conditions of 35% to 50% solvent A over 30 min at 0.5 mL/min, 10ºC. Elution of products was monitored spectrophotometrically at 210 nm, 234 nm, 250 nm and 270 nm. Samples were prepared in acetonitrile and injection volumes of 1 to 10 µL were used. Reproducibility was confirmed by repeat injections. Data was processed using Agilent Technologies ChemStation for LC 3D system software (B.04.03).

Methylation

After extraction of reaction products by SPE or solvent extraction and evaporation of the solvent, 200 µl methanol and 200 µl (trimethylsilyl)diazomethane (2 M in diethyl ether) were added and the reaction mixture kept at room temperature for one hour in a sealed vial. The solvent was evaporated and products dissolved in 1-3 ml heptane for extraction by SPE. An LC-Si cartridge (Supelclean LC-Si SPE cartridges, 6 mL, 1 g; Supelco Analytical) was conditioned with 3 column volumes of heptane before the sample mixture was loaded. The SPE cartridge was washed with 1 column volume of

heptane and dried under vacuum for 5 to 15 min. The methylated products were eluted with 1 column volume of isopropanol. After evaporation of the solvent under nitrogen gas or under vacuum, the sample was diluted in 1 ml acetonitrile and analyzed by chiral RP-HPLC. The synthesized racemic product mixture (see below) and standards of 7*S*,17*S*-dihydroxy DHA and 10*S*,17*S*-dihydroxy DHA were methylated using the same procedure prior to chiral RP-HPLC.

Synthesis of racemic dihydroxy DHA mixture

To develop the chiral RP-HPLC separations, a racemic mixture of 7,17-dihydroxy DHA and 10,17-dihydroxy DHA isomers was generated from the enzymatic reaction product mixture, by oxidizing the hydroxy groups to ketones then reducing them back to hydroxy groups. Briefly, the extracted enzymatic reaction products (~0.7 mg) were dissolved in 1 mL of dry dichloromethane in a round bottom flask and ~10 mg of Dess-Martin periodinane (DMP) was added. Before use, the DMP was purified by dissolving in dichloromethane and filtering to remove insoluble 2-iodoxybenzoic acid (IBX). The reaction mixture was sealed and stirred for 20 min at room temperature, to form a cloudy solution. Completion of the reaction was monitored by TLC, using a solvent system of *n*-heptane/diethyl ether/acetic acid (40:60:1). Complete conversion of starting material (R_f 0.1) to oxidized ketone products (R_f 0.2) was observed. The solvent was evaporated under nitrogen and the products were dissolved in ethyl acetate (4 mL). The reaction products were washed with two volumes of sodium thiosulfate (100 g/L), stirring for 10 min each, followed by two volumes of water and dried over anhydrous sodium sulfate. The isolated oxidized product was yellow. The solvent was filtered and evaporated under nitrogen.

The oxidized products were then dissolved in 1 mL methanol and 10 mL water was added. Sodium borohydride in water (1 M, 200 µL) was added and the mixture stirred for 5-15 min to reduce the oxidized products. The yellow color disappeared within 5 min. The reaction was stopped by adding 100 µL glacial acetic acid. The reduced products were extracted with 2 × 20 mL diethyl ether and the solvent was evaporated under vacuum. Completion of the reaction was confirmed by TLC using the same solvent system as above.

RESULTS

Conversion of DHA to dihydroxy-DHA using soy flour

Based on previous work using soy flour as a lipoxygenase source for the synthesis of hydroperoxy conjugated octadecaenoic acids (see Table 1) and optimized conditions for the enzymatic synthesis of lipid mediator analogues from LC-PUFAs catalyzed by a commercial 15-sLOX-1 [34], soy flour was tested as a lipoxygenase source for the enzymatic synthesis of dihydroxy-DHA isomers from DHA without any protein purification. The following initial conditions were used: 100 µM DHA, 25 µL soy flour solution (1 g soy flour in 20 mL water), 10 mL phosphate buffer (50 mM, pH 7), room temperature, and 15 min reaction time. Under these conditions, three major products were detected in the reaction mixture. Based on comparison with the products of the reaction of DHA catalyzed by commercial 15-sLOX-1 by NP-HPLC (retention time and absorption spectra), these products were tentatively identified as a monohydroxy product, 17*S*-hydroxydocosahexa-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-enoic acid (17*S*-HDHA), and two dihydroxy products, 7*S*,17*S*-dihydroxydocosahexa-4*Z*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-enoic acid (7*S*,17*S*-diHDHA; RvD5) and 10*S*,17*S*-dihydroxydocosahexa-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-enoic acid (10*S*,17*S*-diHDHA; PDX) [34].

Colorimetric detection of LOX in soy flour

To determine which lipoxygenase isozymes were present in the soy flour, a series of colorimetric tests developed by Suda et al. [33] were applied to the soy flour solution. Results for the tests are shown in Table 2. These tests indicated the presence of all three LOX isozymes in the soy flour solution. Similarly, a commercial 15-sLOX-1 used for comparison was also tested and the results are shown in Table 2. As expected, a rapid response was observed for Test I, confirming the presence of this isozyme. The result for Test II was inconclusive; bleaching of the solution was observed, but not within the 5 minute time frame stipulated by Suda et al. [33], suggesting that if LOX-2 is present, the concentration and/or activity is low. Test III indicated that the commercial LOX-1 solution also

contained LOX-3. The commercial preparation was labeled as partially purified, so it is possible that multiple isozymes are present.

Optimisation for production of dihydro(pero)xy-DHA

The initial experiment showed that soy flour could be used as an enzyme source for the conversion of DHA into dihydroxy lipid mediators. The reaction was optimized to maximize yield of the dihydroxy products.

pH and soy flour solution volume

pH is important in lipoxygenase catalysis [8,34-36]. A pH range of 5.0 to 11.0 was studied using three different buffers (phosphate, Tris-HCl and tetraborate). At low pH (pH 5.0 – 6.5) using 25 µL soy flour solution, the formation of all products was suppressed. The synthesis of monohydroxy-DHA compounds increased significantly with increasing pH, whilst the formation of dihydroxy products reached a maximum at pH 7.5. Only a small amount of DHA (≤5%) remained under these conditions. As there were significant amounts of monohydroxy-DHA remaining that could potentially be converted into dihydroxy-DHA products, the optimization was continued using increasing amounts of the soy flour solution (100 µL – 400 µL) over a smaller pH range (pH 7.0 – 10.0; Figure 1). At higher soy flour amounts (100 µL and above), the optimum pH for the production of dihydroxy-DHA compounds increased to 8.0. Dihdyroxy-DHA product formation significantly increased with increasing soy flour solution volume up to 300 µL, then began to decrease at 400 µL. Monohydroxy-DHA product formation decreased with increasing soy flour solution volume, as it was being converted into dihydroxy-DHA products. At pH 8.0 with 300 µL soy flour solution, no DHA remained and very little monohydroxy-DHA remained. At higher pH (>8.5) monohydroxy-DHA products were still formed. Phosphate buffer gave the highest yield of dihydroxy-DHA products. Optimum conditions found were: phosphate buffer (50 mM), pH 8 and 300 µL soy flour solution.

Substrate concentration

DHA concentration was optimized in the range 0.025 mM to 1.0 mM, using 300 µL soy flour solution and 10 mL phosphate buffer (pH 8, 50 mM). Increasing the substrate concentration increases the

amount of product that can potentially be formed, but can also lead to substrate and/or product inhibition of the enzyme [10,14,19,37-39], and the development of anaerobic conditions [34]. Under the conditions studied, the formation of dihydroxy-DHA products increased significantly with increasing DHA concentration, reaching a maximum at 0.2 mM (Figure 2). At concentrations above 0.2 mM, dihydroxy product formation rapidly decreased, whilst the production of 17-HDHA increased and at 0.5 mM DHA and above, very little dihydroxy-DHA was detected.

At high DHA concentrations (≥ 0.5 mM), increasing amounts of 17-HDHA remained in the reaction mixture and two unknown products were also detected. The first eluted earlier than DHA with λ_{max} at 240 nm, and the second eluted after the monohydroxy-DHA products but before the dihydroxy-DHA compounds with λ_{max} at 235 nm. These compounds may have been formed through the lipohydroperoxidase activity of the lipoxygenase enzyme, which can occur under anaerobic conditions [40-42]. Increasing the concentration of DHA can increase the rate and overall total consumption of oxygen, leading to anaerobic conditions. The optimum DHA concentration for the production of dihydroxy-DHA compounds was found to be 0.2 mM. At this concentration, a small amount of 17-HDHA still remained.

Surfactants

Surfactants have previously been used to improve substrate solubility and enhance lipoxygenase product formation [43-45]. Two non-ionic surfactants that have been previously utilized in lipoxygenase reactions, Triton X-100 (primarily used to solubilize and extract the enzyme [46,47]) and Tween 20 (commonly used to solubilize the substrate [44,45,48-50]), were tested for their ability to increase the yield of dihydroxy-DHA products in this reaction. Surfactants were tested in the concentration range 0.001 to 0.1% m/v. The addition of Triton X-100 resulted in an increase in the production of both dihydroxy-DHA isomers, with best results at a Triton X-100 concentration of 0.01% m/v to 0.05% m/v. 17-HDHA concentration increased with increasing Triton X-100 concentration. Tween 20 also caused an increase in production of dihydroxy-DHA compounds, but Triton X-100 (0.01% m/v) was selected as the preferred surfactant as it gave the largest increase in product yield.

Time

To investigate the reaction time needed to obtain the largest yield of dihydroxy-DHA products, large-scale reactions (100 mL) were performed and 10 mL aliquots were taken at various time points (1 min to 180 min) to monitor the course of the reaction. Production of the dihydroxy-DHA compounds was rapid with significant amounts formed after only one minute, and production continuing for 20 to 30 min. The concentration of 7,17-diHDHA reached a maximum at 20 min and 10,17-diHDHA at 30 min. Although there were still significant amounts of 17-HDHA remaining and available for conversion, after 30 min the concentration of both dihydroxy-DHA compounds gradually decreased. Thirty minutes was chosen as the optimum reaction time.

Oxygen

As oxygen is a substrate in lipoxygenase catalyzed reactions, the concentration of oxygen has a significant effect on the reaction. The oxygen concentration in the buffer was altered by bubbling gases through the buffer for 5 min before initiating the reaction. Nitrogen was used to remove oxygen from solution, air to ensure air saturation the buffer (dissolved oxygen ~ 8.5 mg/L, 0.27 mM), and oxygen to fully oxygenate the solution (dissolved oxygen ~ 35 mg/L, 1.1 mM). Reactions were performed in a sealed environment, using a balloon to maintain positive pressure of the relevant gas. Increasing the concentration of oxygen in the reaction resulted in an increase in both monohydroxy- and dihydroxy-DHA products by approximately 16% and 30% respectively. Removing oxygen from solution suppressed the formation of all dioxygenation products, and instead resulted in the synthesis of anaerobic reaction products, also observed in reactions at high substrate concentrations. Saturating the buffer with air had no effect. Further experiments showed that sealing the reaction environment was not required.

The oxygen saturated buffer reaction also had a higher concentration of 17-HDHA remaining, which could potentially be converted into dihydroxy-DHA products, therefore, the volume of soy flour solution was re-examined over the range 300 to 1000 µL. Increasing the soy flour solution volume resulted in a significant increase in the amount of dihydroxy-DHA compounds produced, to a maximum at 800 µL soy flour solution, and a reduction in the amount of 17-HDHA remaining in the

reaction. 10,17-diHDHA increased by approximately 60%, and 7,17-diHDHA increased by approximately 35%. The re-evaluated optimum conditions found were oxygen saturated buffer and 800 µL soy flour solution.

Temperature

Temperature can have a significant effect on enzyme-catalyzed reactions; it may affect reaction rate, enzyme specificity, enzyme structure, solubility of substrates and mass transfer [51]. The reaction was optimized over the temperature range 0ºC to 40ºC. Production of dihydroxy-DHA products increased with temperature to a maximum at ambient temperature (21ºC to 23ºC), then decreased as temperature increased further. From 0ºC to 21ºC, the concentration of 10,17-diHDHA increased more than two-fold, whilst the concentration of 7,17-diHDHA increased by 40%. There was little change in the small amount of 17-HDHA detected across the temperature range. At 40ºC, yield of all products was lowest and approximately 15% of the DHA remained unreacted at the end of the reaction. There was no increase in non-enzymatic products observed when temperature was increased. Ambient temperature was chosen for further experiments.

Reproducibility

The final optimized conditions were found to be: 800 µL of soy flour solution (1 g / 20 mL), 0.2 mM DHA, phosphate buffer (pH 8, 50 mM), oxygen saturation (~ 1.1 mM), Triton X-100 (0.01% m/v), ambient temperature, 30 min reaction time. The reproducibility of the optimized reaction was measured by repeating the reaction five times on three different days (Figure 3). The relative standard deviation (RSD) for 7,17-diHDHA was 2.5%-3.7% for each of the three days, and for 10,17-diHDHA was 3.0%-6.0%. The RSDs for the combined data of the 15 reactions across three days were 3.2% and 4.8% for 7,17-diHDHA and 10,17-diHDHA, respectively.

Yield

The yield of the reaction was estimated from the NP-HPLC peak intensities relative to the internal standard. Molar extinction coefficients were used to correct for differences in UV absorbance: PGB_2 (280 nm) 30 000 M^{-1} cm^{-1} [52]; 7*S*,17*S*-diHDHA (243 nm) 33 500 M^{-1} cm^{-1} [53]; and 10*S*,17*S*-

diHDHA (270 nm) 38 000 M^{-1} cm^{-1} [54]. The estimated yield for the optimized reaction was 25% 7*S*,17*S*-diHDHA and 48% 10*S*,17*S*-diHDHA.

Product characterization

The major products of the soy flour catalyzed reaction; 17-HDHA, 7,17-diHDHA and 10,17-diHDHA, were identified by comparison of the NP-HPLC retention times and UV-visible absorbance spectra with the known products of the 15-sLOX-1 catalyzed DHA reaction [34]. To confirm the compound identities, the products were also characterized by GC-MS and chiral RP-HPLC.

GC-MS

The reduced reaction products were fully hydrogenated and derivatized to trimethylsilyl derivatives prior to GC-MS analysis. The results are summarized in Table 3 and confirmed the presence of 17-HDHA, 7,17-diHDHA and 10,17-diHDHA. An additional mono-hydroxy product, 10-HDHA, was also detected by GC-MS, but was not identified by NP-HPLC.

Chiral RP-HPLC

Soybean lipoxygenase is known to produce compounds with *S*-chirality, although the specificity of this reaction can vary depending on reaction conditions [50,55-57]. To determine if this chiral specificity was maintained when using soy flour as a lipoxygenase source, the chirality of the products was determined by chiral RP-HPLC. Prior to analysis, the compounds were methylated to increase retention and improve chromatographic behavior. A racemic mixture of four 7,17-diHDHA and four 10,17-diHDHA stereoisomers was synthesized to ensure all compounds could be distinguished. The methyl esters of 7,17-diHDHA from the soy flour LOX catalyzed reaction co-eluted with a 7*S*,17*S*-diHDHA standard and 10,17-diHDHA from the soy flour LOX catalyzed reaction co-eluted with a 10*S*,17*S*-diHDHA standard, confirming the *S*,*S* chirality of both compounds. We did not detect any of the other stereoisomers for either compound in the reaction mixture at concentrations above 0.5% of the major product, demonstrating the high stereoselectivity of the reaction. Chiral RP-HPLC chromatograms are included in the supplementary material (Supplementary Figures 1-4).

Comparison to commercial / purified LOX

The results have confirmed that a simple soy flour solution can act as a source of lipoxygenase to catalyze the double dioxygenation of DHA. The reaction has been shown to synthesize the same compounds that are produced by a commercial 15-sLOX-1 [34]. A comparison of reaction conditions is shown in Table 4. By comparing the yield from the optimized reaction of this study with the yield achieved using previously optimized conditions for the 15-sLOX-1 reaction [34], it was found that both reactions produced very similar amounts of the two dihydroxy-DHA isomers (Figure 4, normalized for the amount of DHA used). As it was possible to use higher concentrations of DHA with the soy flour solution (0.2 mM compared to 0.1 mM), the same volume reaction was able to produce twice as much of the two dihydroxy products as the commercial enzyme reaction.

Application to other substrates

The optimized conditions for the soy flour LOX catalyzed reaction with DHA were also applied to other biologically relevant substrates (EPA; *all-cis*-eicosa-5,8,11,14-tetraenoic acid, arachidonic acid, AA; *all-cis*-docosa-7,10,13,16,19-pentaenoic acid, DPAn-3; and *all-cis*-docosa-4,7,10,13,16-pentaenoic acid, DPAn-6), the results are shown in Figure 5. The soy flour LOX was able to catalyze reactions with all substrates, producing compounds analogous to those synthesized from DHA. In each case, there was no substrate and very little monohydroxy product remaining at the end of the reaction time. The major difference seen between the reactivity with the different substrates was the ratios of the two dihydroxy isomers. For EPA, similar amounts of both isomers were formed; for DHA and AA, the ratio was approximately 2.5:1 in favor of the dihydroxy isomer with the hydroxy groups separated by 7 carbons; for DPAn-6, the ratio was approximately 7:1 also in favor of this isomer; whilst the DPAn-3 reaction was the only one to produce more of the dihydroxy isomer with the hydroxy groups separated by 10 carbons, with a ratio of approximately 15:1. This is similar to results previously observed with the commercial 15-sLOX-1 reaction [34].

DISCUSSION

Whilst previous work has demonstrated the potential of simple soy flour preparations to convert eighteen carbon fatty acids (primarily linoleic and linolenic acids) to their hydroxy analogues (see Table 1), we have shown that soy flour lipoxygenase can also be used to convert omega-3 and omega-6 PUFAs into mono- and di-hydroxy fatty acid resolvin analogues. Colorimetric screening indicated that three LOX isozymes (1, 2 and 3) were active in the aqueous soy flour solution, with LOX-2 appearing significantly more active and/or higher in concentration than in a commercial LOX-1 preparation. This is consistent with previous findings that LOX-2 is unstable after purification, but activity can be preserved within the flour matrix [15].

The LOX isozymes (1, 2 and 3) each have different optimum pHs (approximately 9.0, 6.5 and 6.0, respectively) [35,36,58], however, the optimum pH found in this study for the conversion of DHA into dihydroxy-DHA was pH 8.0. Previous work using simple soy flour preparations has generally been undertaken at pH 9-10 (for free fatty acid substrates) [13,14,16-20,23,24,26] or around pH 7 (for esterified substrates) [15,22,25]. We propose that pH 8.0 used in our study is not the optimum for any of the individual LOX isozymes present in the soy flour, but may reflect a compromise to maximise the activity of multiple LOX isozymes in the reaction, suggesting that more than one LOX is active. It has also been found that the optimum pH for the second hydroperoxidation is lower than the first (pH 7.5-8.0 compared to pH 9.0) when using LOX-1 [35,48], which may further explain the optimum pH found in this study.

Maximum conversion of DHA into dihydroxy-DHA products was achieved at a substrate concentration of 0.2 mM. At lower substrate concentrations, less product was formed presumably due to the limited amount of substrate available, whilst at higher substrate concentrations, the amount of dihydroxy-DHA produced did not remain constant, instead it decreased significantly. This could be attributed to a number of factors: i) insufficient oxygen; ii) substrate inhibition; and iii) product inhibition. To fully convert 0.2 mM DHA into dihydroxy-DHA products, 0.4 mM oxygen concentration is required; whilst the oxygen concentration in air-saturated water at 20ºC is

approximately 0.29 mM [59]. Additional oxygen can enter the solution during the reaction, but at these concentrations, oxygen will be the limiting reagent and may lead to anaerobic reaction conditions. Under anaerobic conditions, the lipoxygenase enzyme can exhibit lipohydroperoxidase activity resulting in the formation of new products [40-42]; the formation of new products was observed in this study at high substrate concentrations. Product hydroperoxides are required for enzyme activity as they convert the inactive Fe(II) form of the enzyme to the catalytically active Fe(III) form [60,61]. However, lipoxygenase enzymes have been found to be inhibited by both substrate [14,37-39,60,62] and product [10,19,60,62]. The free enzyme has a much higher affinity for the product than for the substrate[10,39], so high product concentrations can prevent substrate binding and cause inhibition. Whilst high substrate levels, especially initially, can prevent hydroperoxide binding needed for enzyme activation [37,62].

The production of dihydroxy-DHA isomers increased with increasing soy flour (and therefore enzyme) concentration. This was expected due to the requirement to have sufficient enzyme present to transform the DHA before inactivation due to product inhibition, and because the kinetics of the second hydroperoxidation are significantly slower than the first [34,63]. However, further increases in soy flour concentration caused a decrease in reaction products, possibly due to other components in the soy flour interfering or inhibiting the reaction, increased rate of consumption of oxygen leading to anaerobic conditions, and product inhibition.

The reaction started rapidly upon addition of the soy flour extract, however, the reaction was slower than that reported previously using 15-sLOX-1 [34]. After reaching maximum yield at 20-30 min, the reaction products began to decrease. This decrease was not reported for the reaction with 15-sLOX-1 [34], which may suggest that additional enzymes which are able to metabolize the dihydroxy-DHA reaction products are also present and active in the soy flour extract, such as hydroperoxide isomerases and hydroperoxide lyases [64]. Anaerobic conditions can also result in degradation of hydroperoxides through lipoxygenase lipohydroperoxidase activity [40-42]. The importance of oxygen in this reaction was further demonstrated in the oxygenation experiments; increasing the concentration of oxygen resulted in the production of more dihydroxy-DHA, and the reduction of

oxygen caused a decrease in products through activation of the anaerobic lipoxygenase pathway [40-42].

Srinivasulu and Rao previously found that surfactants could enhance LOX activity at low concentrations, but would inhibit LOX activity at higher concentrations [44]. In our study, no inhibition was observed, but the enhancement was only effective in a limited concentration range. Enhancement of LOX activity may be due to stabilization of protein structure [44], increased thermal stability of LOX [65], shortening of the induction period [66,67] (which can be removed by the addition of hydroperoxides), and increasing the solubility of fatty acid substrates [45]. It has been proposed that inhibition and enhancement observed from the addition of surfactants is a result of changes in the effective concentration of substrate, due to partitioning of the substrate into surfactant micelles, which significantly affects enzyme activity due to enzyme preference for non-micellar substrate [45]. The formation of emulsions can also hinder the extraction of reaction products resulting in lower apparent yields.

Overall yield was affected by temperature; at both low and high temperatures enzyme activity was decreased. The solubility of both oxygen and substrate in water is temperature dependent, with oxygen solubility decreasing with increasing temperature and substrate solubility increasing [18]. Gradual loss of lipoxygenase activity has been reported at higher temperatures [68]. Although some previous studies have suggested that lipoxygenase-catalyzed reactions should be carried out at low temperatures to prevent the formation of nonspecific products, this was not observed in this study and was consistent with our previous study using 15-sLOX-1 and DHA [34]. Overall, the optimum temperature for the reaction is a compromise between increased lipoxygenase activity and stability at lower temperatures and improved mass transfer and miscibility at higher temperatures [25].

Simple soy flour preparations have been used previously as a source of lipoxygenase for various reactions (see Table 1). However, the work presented in this study is the first time a simple soy flour preparation has been used for the transformation of 20 carbon and 22 carbon fatty acids, and the first time it has been used to synthesize dihydroperoxy fatty acid products.

Soy flour is a feasible alternative to purified lipoxygenase enzymes for the conversion of PUFAs into dihydroxy fatty acid lipid mediator analogues. Characterization of the reaction products confirmed the soy flour lipoxygenase has high regio- and stereo-selectivity, comparable to a commercial lipoxygenase preparation. The yield for the conversion of DHA was equivalent to that achieved with commercial 15-sLOX-1, but higher concentrations of substrate could be used with the soy flour preparation, resulting in more product from the same reaction volume. Similar to the commercial 15-sLOX-1, the soy flour preparation could also be used to transform a range of other PUFA substrates. Whilst two dihydroxy isomers were detected with each of the five substrates, for the two DPA substrates, one isomer was formed in significantly larger amounts than the other. This has also been observed with 15-sLOX-1 [34]. The reason for this difference is currently unknown. Based on the cost difference between the commercially available 15-sLOX-1 and soy flour, we estimate that using soy flour as an alternative lipoxygenase source is more than 28 000 times cheaper for the production of dihydroxy-DHA compounds.

CONCLUSIONS

We have demonstrated, for the first time, that soy flour can be used as an alternative to commercial and purified LOX preparations for the conversion of biologically important omega-3 and omega-6 PUFAs into mono- and dihydroxy fatty acid resolvin analogues. The reaction conditions are mild and the conversion is rapid. The regio- and stereo-selectivity of the reaction is comparable to a commercial LOX preparation, with improved yield and the reaction is reproducible. This method has potential to be used for the production of these compounds for pharmaceutical and nutraceutical purposes at much lower cost than using purified enzymes or total chemical synthesis methods.

ACKNOWLEDGEMENTS

Dr Adcock is the recipient of an Australian Research Council Discovery Early Career Researcher Award (project number DE140100162).

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Table 1. *Reports of simple soy flour preparations used for the synthesis of lipid hydroperoxides*

Substrate	pH	Purpose	Year	Ref.
Safflower and soybean oil soap	9 – 10	Synthesis of hydroxy conjugated octadecadienoic acids	1971	[14]
Trilinolein, linoleic acid, sunflower oil	9.0	Testing ability to directly oxidize TAGs	1993	[15]
Linoleic and linolenic acid	9.5	Production of natural green note flavor compounds	1995	[16]
Linoleic acid, linolenic acid	9.0 – 9.5	Green note compound synthesis (aliphatic alcohols and aldehydes)	1995	[17]
Hydrolyzed safflower oil	10	Large scale production of 13-HPOD from hydrolyzed safflower oil	1996	[18]
Linoleic acid in hydrolyzed safflower oil	10	Synthesis of lipid hydroperoxides, for conversion to hydroxides for fine chemicals	1998	[19]
Hydrolyzed safflower and linseed oils	8.25	Green note compound synthesis (hexanal and hexenal)	2002	[20]
Linolenic acid	6 or 8.7	Green note compound synthesis	2005	[21]
Butterolein	7.0	Enzymatic synthesis of C6-C10 flavor aldehydes	2006	[22]
Hydrolyzed linseed oil	9.5	Development of a method for real time reaction monitoring	2007	[23]
Hydrolyzed linseed oil	9.0	Synthesis of *cis*-3-hexenal	2008	[24]
Soybean oil	6	Industrial-scale production of hydroperoxides	2011	[25]
Hydrolyzed linseed oil	9.3	Preparing lipid hydroperoxide substrate for hydroperoxide lyase	2013	[26]
Linoleic acid	9.0	Testing methods for protein extraction from soy flour	2014	[13]

Table 2. *Results of colorimetric tests for the detection of lipoxygenase isozymes.*

Test	Soy flour amount (mg)	Bleaching Start (min)	Bleaching End (min)	Result	15-sLOX-1 amount (µg)	Bleaching Start (min)	Bleaching End (min)	Result
I: LOX-1	2.5	0.5	1.5 – 2	Positive	140	0.5	0.75 – 1	Positive
II: LOX-2	5.0	3 – 5	9 – 10	Positive	280	10 – 15	20 – 25	Inconclusive
III: LOX-3	2.5	0.5	3.5 - 4	Positive	140	0.5	2.5 – 3	Positive

Table 3. *Characteristic m/z fragments detected by GC-MS of the hydrogenated, trimethylsilyl derivatives of the products from the soy flour lipoxygenase catalysed dioxygenation of DHA.*

Retention time (min)	**Compound**	***m/z* fragments**
26.62	10-HDHA	73, 117, 271, 309, 331, 395, 485
26.94	17-HDHA	73, 117, 173, 309, 395, 429, 485
28.81	10,17-diHDHA	73, 117, 173, 331, 359, 427, 517, 573
28.85	7,17-diHDHA	73, 117, 173, 289, 402, 427, 517, 573

Table 4. *Comparison of reaction conditions for the dioxygenation of DHA catalyzed by soy flour lipoxygenase and commercial 15-sLOX-1 (10 mL reaction)*

Variable	**Soy flour**	**15-sLOX-1 [34]**
DHA	0.2 mM	0.1 mM
Enzyme	40 mg soy flour (800 µL)	5 mg
pH	8.0	9.0
Buffer	Phosphate, 50 mM	Tetraborate, 50 mM
Temperature	Ambient	Ambient
Time	30 min	15 min
O_2	Oxygen sparging	No sparging
Surfactant	Triton X-100, 0.01% m/v	None

FIGURE CAPTIONS

Figure 1. Optimization of buffer pH (phosphate buffer) and soy flour solution volume for the production of 10,17-dihydroxy DHA from the dioxygenation of DHA catalyzed by soy flour lipoxygenase. Peak area measured by NP-HPLC and corrected against the internal standard PGB2.

Figure 2. Effect of DHA concentration on the formation of dihydroxy-DHA products in the dioxygenation of DHA catalyzed by soy flour lipoxygenase. Peak area measured by NP-HPLC and corrected against the internal standard PGB2.

Figure 3. Reproducibility of the optimized soy flour lipoxygenase catalyzed reaction with DHA, performed five times per day for three days. Peak area measured by NP-HPLC and corrected against the internal standard PGB2; error bars represent one standard deviation.

Figure 4. Comparison of product yields for the dioxygenation of DHA catalyzed by soy flour lipoxygenase and commercial 15-sLOX-1. Peak area measured by NP-HPLC and corrected against the internal standard PGB2; results normalized to milligrams of DHA used; error bars represent one standard deviation.

Figure 5. Comparison of reaction products formed in the soy flour lipoxygenase catalyzed dioxygenation of different PUFA substrates. Peak area measured by NP-HPLC and corrected against the internal standard PGB2.

Figure 1.

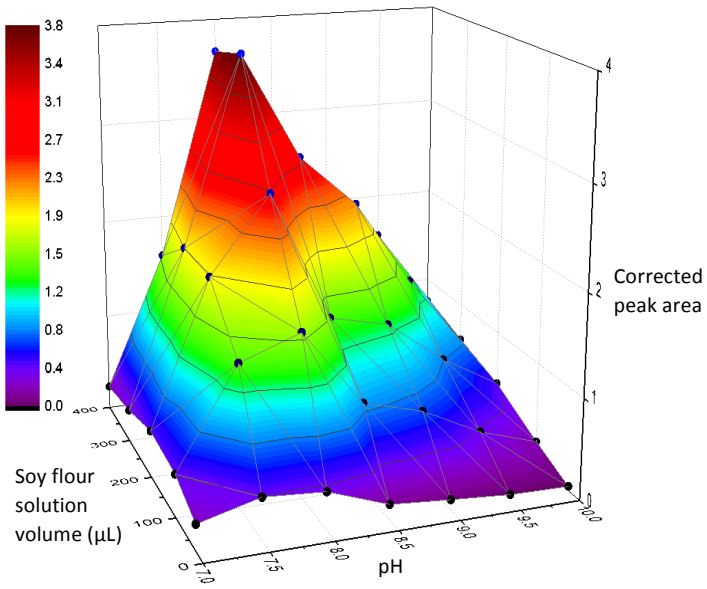

3.8
3.4
3.1
2.7
2.3
1.9
1.5
1.2
0.8
0.4
0.0
400
300
200
100
0
Soy flour solution volume (µL)
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH
4
3
2
1
0
Corrected peak area

Figure 2.

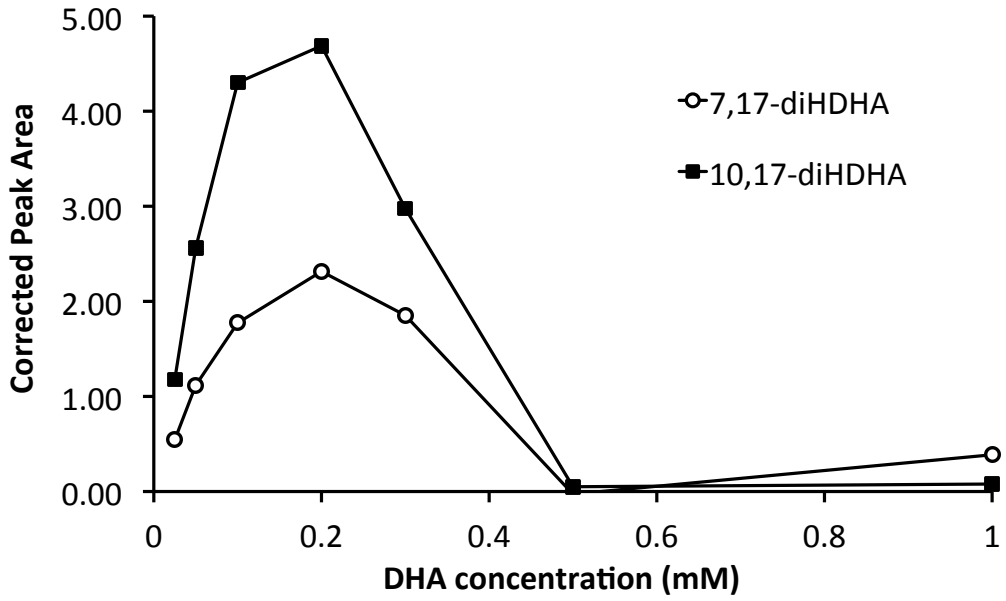
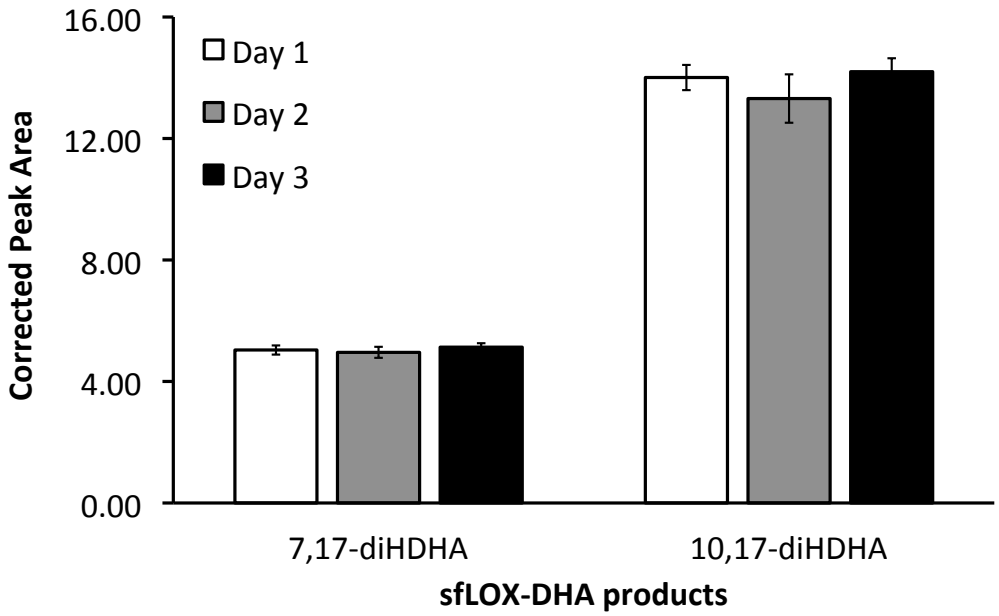

5.00
4.00
3.00
2.00
1.00
0.00
Corrected Peak Area
0
0.2
0.4
0.6
0.8
1
DHA concentration (mM)
7,17-diHDHA
10,17-diHDHA

Figure 3.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4.00
0.00
Corrected Peak Area
Day 1
Day 2
Day 3
7,17-diHDHA
10,17-diHDHA
sfLOX-DHA products

Figure 4.

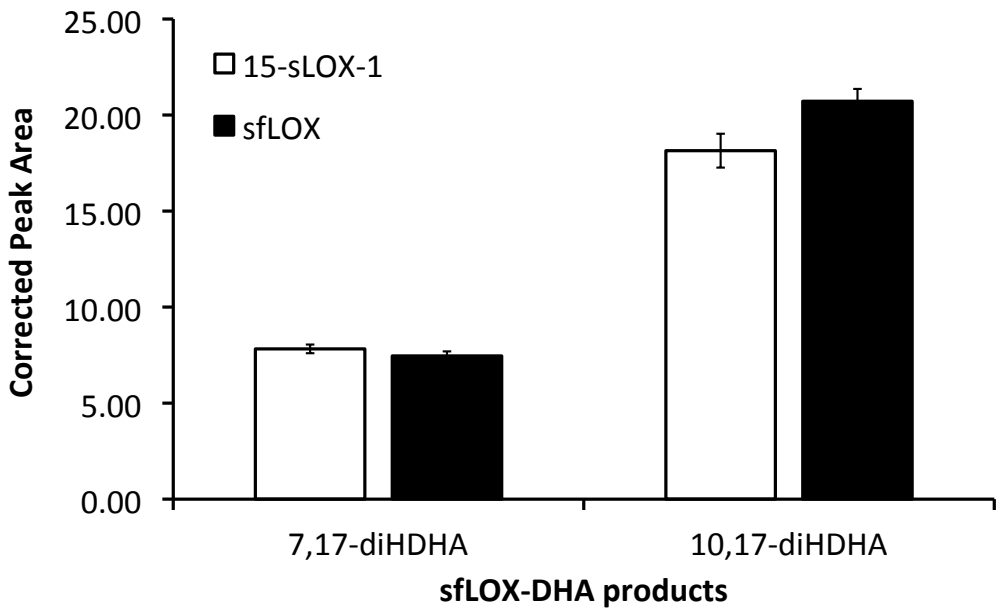
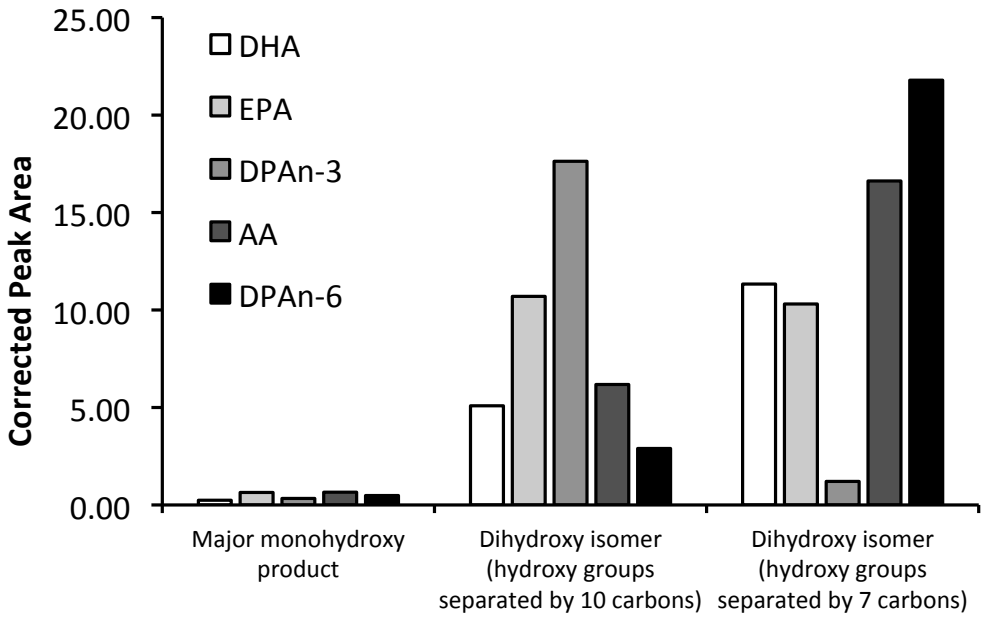

25.00
20.00
15.00
10.00
5.00
0.00
15-sLOX-1
sfLOX
Corrected Peak Area
7,17-diHDHA
10,17-diHDHA
sfLOX-DHA products

Figure 5.


25.00
20.00
15.00
10.00
5.00
0.00
Corrected Peak Area
DHA
EPA
DPAn-3
AA
DPAn-6
Major monohydroxy product
Dihydroxy isomer (hydroxy groups separated by 10 carbons)
Dihydroxy isomer (hydroxy groups separated by 7 carbons)