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Lyophilized extracts from vegetable flours as valuable alternatives to purified oxygenases for the synthesis of oxylipins

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ABSTRACT

In this work, the whole aqueous extracts of soybean flour and oat flour have been used as valuable alternatives to purified oxygenase enzymes for the preparation of oxylipins derived from (5Z,8Z,11Z,14Z,17Z)-eicosapentaenoic acid (EPA). The lipoxygenase activity in the aqueous extracts of soybean (*Glycine* max. L.) flour was monitored with linoleic acid as substrate and compared with the commercially available purified enzyme (LOX-1). Oat flour extracts (*Avena sativa* L.) were evaluated for their peroxygenase activity by comparing different enzyme preparations in the epoxidation of methyl oleate. It was found that lyophilization of the aqueous extracts from these vegetable flours offers advantages in terms of enzyme stability, reproducibility and applicability to preparative organic synthesis. The lyophilized enzyme preparations were tested for the oxyfunctionalization of EPA and the formed products were isolated in satisfactory yields. In the presence of lyophilized extract from soybean, EPA gave 15*S*-hydroxy-(5*Z*,8*Z*,11*Z*,13*E*,17*Z*)-eicosapentaenoic acid in enantiopure form as exclusive product. Peroxygenase from oat flour was less selective and catalyzed the formation of different epoxides of EPA. However, the biocatalyzed epoxidation of EPA under controlled conditions allowed to obtain optically active (17*R*,18*S*)-epoxy-(5Z,8Z,11Z,14Z)-eicosatetraenoic acid (65% *ee*) as the main monoepoxide, among the five possible ones.

1. Introduction

Oxylipins are a large group of oxygenated metabolites of polyunsaturated fatty acids (PUFAs), that include fatty acid hydroperoxides, hydroxy-, epoxy- and keto-derivatives, divinyl ethers, volatile aldehydes, and the plant hormone jasmonic acid [1,2]. These molecules, which are ubiquitously present in eukariots, display important pro-inflammatory or anti-inflammatory effects in animals [3,4], whereas in plants they are involved in a variety of signalling functions as well as in the resistance to microbial pathogens [5,6].

The *in vivo* biosynthesis of oxylipins involves a variety of enzymes, belonging to the class of dioxygenases or monooxygenases, with specificity for the different positions of the polyene systems of PUFAs. In this context, most of investigations have been focused on oxylipins derived from linoleic acid, arachidonic acid and ω-3 fatty acids, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, due to the importance of their physiological effects as lipid mediators of inflammation with a potential role in metabolic diseases [7,8] and, more recently, as cancer preventing agents [9–11].

A common starting step in the biosynthesis of oxylipins is the lipoxygenase-catalyzed formation of a hydroperoxide of the fatty acid, whose subsequent conversion through different enzymatic pathways results in a variety of oxygenated derivatives.

Due to the increasing interest in the biological effects of ω-3 fatty acids and related compounds there is the need to synthesize oxylipins in preparative scale. Considerable synthetic efforts have been devoted to the development of selective processes for the production of single isomers of hydroxy- or epoxy- fatty acids, but in most cases multistep sequences are necessary to control the functionalization of a specific double bond of the polyenic molecules [12–14].

Two different approaches have been applied (Scheme 1), the first of which is based on the stereoselective construction of the (Z,Z)-1,4-diene system by Z-selective Wittig reactions or by C—C coupling of suitably functionalized fragments followed by Lindlar reduction [15]. Strong bases and metal catalysts are used and isomerization of the skipped Z-olefins or over reduction often occur as side reactions. In the second approach, PUFAs are used as individual starting materials for subsequent modifications [16] and in many cases the selective epoxidation of the double bond closest or far away to the carboxylic acid group has been exploited [17] for the access to a variety of products through oxidative cleavage of the epoxides, isomerization to allylic alcohol or ring opening.

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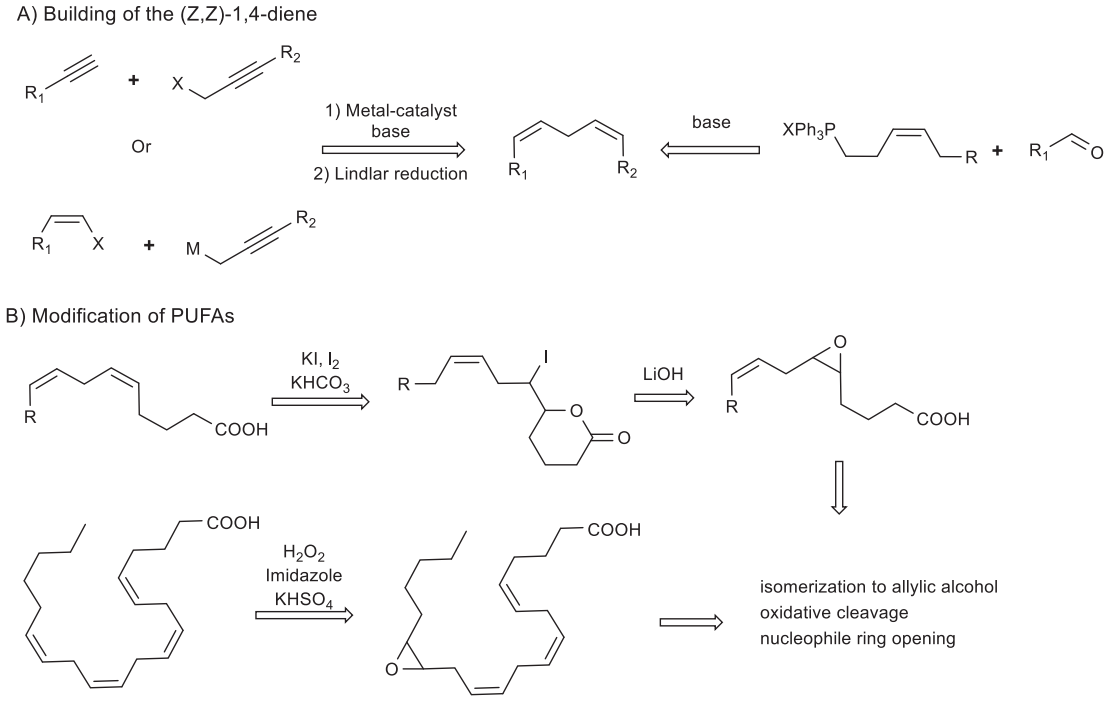


Scheme 1. General methods for the chemical synthesis of functionalized PUFAs.

Enzyme-catalyzed processes, which are usually performed in an aqueous medium and under mild temperature conditions, offer a valuable alternative in terms of selectivity and sustainability, also for their reduced demand of reagents, required for protection-deprotection of substrates, and decreased purification steps. In the context of PUFA modifications, oxygenases are the enzymes of choice but, unfortunately, their commercial availability in purified form is rather limited and currently too expensive for their application in synthesis. In many cases these enzymes have been isolated in laboratory scale from vegetables [18–20], algae [21,22], fungi [23,24] and bacteria [25,26] for their characterization in terms of activity and positional specificity in the formation of fatty acid derivatives.

Lipoxygenase from soybean (*Glycine* max) is a non-heme iron-containing enzyme [27] able to catalyse the addition of molecular oxygen across the double bonds of polyunsaturated fatty acids forming their corresponding hydroperoxides through a well established mechanism [28]. Different isozymes are known for soybean lipoxygenase, with a predominant specificity for the 13*S*-position of linoleic acid [29,30], and reactions of the purified isozyme LOX-1 (EC 1.13.11.12) with a variety of fatty acid substrates have been investigated [31–33], in some cases optimizing the conditions to obtain the dioxygenation products [34–36]. The use of soybean flour extracts has been proposed as economical alternative to purified LOX-1 in the derivatization of linoleic acid from vegetable oils [37], and more recently has been applied to DHA [38], but in the latter case the products were not isolated and diluted solutions of substrates were required in order to avoid inhibition of the enzyme.

Among other legumes and cereals, oat (*Avena sativa*) has been taken into consideration for its content in peroxygenase [40] and the whole oat flour extracts, or the corresponding enzyme-enriched microsomal fractions, have been used for the preparation of epoxides of some fatty acids and esters as well as of simple alkenes [41–43], but have not up to date tested on EPA or DHA. Peroxygenases (EC 1.11.2.1) are a class of hydroperoxide-dependent enzymes containing heme that have recently attracted interest for their ability in catalyzing oxyfunctionalization of double bonds, with the advantage over cytochromes to be independent by expensive nicotinamide cofactors [44,45].

In spite of their potential in organic synthesis, the reactions with soybean flour and oat flour extracts have been carried out on analytical scale and the products identified by MS-analysis, without any given information about their isolated yields [37,38,43]. Only a process for the preparative production of hydroperoxide of linoleic acid with soybean flour extract was reported [37]. We then decided to investigate the feasibility of preparative reactions in the presence of flour extracts of soybean or oat using EPA as test substrate. The freeze-drying of flour extracts allowed us to have stable and active enzyme preparations, whose use in the synthesis of oxylipins derived from EPA, coupled with the optimization of reaction and purification conditions, led to good yields of isolated products and here we report the obtained results.

2. Material and methods

2.1. General

(5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-Eicosapentenoic acid (EPA) was obtained by lipase-catalyzed hydrolysis of the corresponding ethyl ester (from Solutex, 90% purity). Lipoxygenase from *Glycine* max (soybean) Type I-B (Lot. SLBS4722, 226,317 U/mg) and immobilized lipase from *Candida antarctica* (Novozym 435) were obtained from Sigma Aldrich. Soybean from organic crops was purchased by “Negozio leggero” shop network. Whole oat seeds from organic crops were available from Ki Group. Lyophilizations were carried out on a Telstar LyoQuest instrument. Centrifugation was carried out on a Thermo Fisher Scientific SL 40R or ALC PK130 centrifuge.

UV-measurements were carried out on a UV–vis VALUE spectrophotometer 8453 (Agilent Technologies). GC analyses were performed on a fast GC Shimadzu 17-A instrument equipped with MS-EI detector (GCMS-QP5050A) on a Supelco SPB-5 capillary column (15 m × 0.1 mm ID × 0.1 μm film thickness). For the analyses the following parameters were set: He flow rate 32.7 mL/min, column inlet pressure 350 KPa, linear velocity 31 cm/sec, split ratio 1:92, injector temperature 250 °C, detector temperature 280 °C. The oven temperature was held at 100 °C for 1 min, then raised to 280 °C at 10 °C/min. MS-EI detection was carried out setting 70 eV ionization voltage, 900 V

electron multiplier voltage and 180 °C ion source temperature. Mass spectra data were acquired in the scan mode in *m/z* range of 40–400.

Chiral HPLC analyses were carried out on a Dionex instrument equipped with a Ultimate 3000 high-pressure binary pump, an ASI-100 autosampler, a TCC-100 thermostated column compartment and a UVD-100 multiple wavelength detector set at 210, 220, 230 and 250 nm. The Chromeleon software (version 6.7) was used for instrument control, data acquisition and data handling.

TLC analyses were performed on aluminum plates coated with silica gel and flourescent indicator F_{254}, revealing the compounds by UV and cerium sulfate.

Column chromatography was performed on silica gel 60 (Merck, 40–63 μm) or Lichroprep Si 60 (Merck, 25–40 μm) using the specified eluents. ^{1}H- and ^{13}C NMR spectra were recorded on Bruker Avance™ 400 spectrometer at 400.13 and 100.62 MHz, respectively. Chemical shifts (δ) are given as ppm relative to the residual solvent peak and coupling constants (*J*) are in Hz. 2D-experiments were carried out using an inverse multinuclear probe with pulse-field Z-gradient and standard Bruker pulse sequence programs. Optical rotations were measured on Jasco DIP-135 polarimeter using a 10 cm length cell.

2.2. Preparation of EPA acid

To a solution of the ethyl ester of EPA (200 mg, 0.6 mmol) in 1.7 mL of dioxane, 300 μL of distilled water and Novozym 435 (200 mg) were added. The mixture was stirred at 55 °C and 280 rpm in a shaker for 3 h. Then, the enzyme was separated by filtration and the solution taken to dryness under reduced pressure. The residue was purified by column chromatography (Si gel 3.9 g, from *n*-hexane to *n*-hexane/EtOAc 9:1 containing 0.1% of HCOOH, 200 mL) to give pure (TLC analysis) EPA (180 mg, 0.59 mmol, 98% yield) as a colorless oil.

2.3. Test for lipoxygenase activity

Air-dried soybean seeds from organic crops were finely grounded with a domestic blender and the obtained flour was sifted on a tea sieve. Defatted flour was prepared by suspending the powder (5 g) in acetone (10 mL) and stirring the mixture for 10 min at room temperature. The suspension was then centrifuged at 2930 *g* and the solution discarded. The procedure was then repeated another two times and the final solid was dried overnight in a fume hood to give 4.4 g of defatted flour.

Defatted soybean flour (1 g) was suspended in 20 mL of distilled water and stirred under magnetic agitation at room temperature for 5, 15 or 30 min. The suspension was then centrifuged at 11,000 *g* for 20 min at 4 °C and the obtained supernatant was used as "soybean extract" sample. The same procedure was also applied to untreated soybean flour, which was extracted for 30 min to give the "not defatted" sample.

Freeze-dried preparation of lipoxygenase was obtained as follows: soybean flour (10 g) was suspended in 100 mL of distilled water and stirred under magnetic agitation at room temperature for 30 min at 1250 rpm. The suspension was then centrifuged at 11,000 *g* for 20 min at 4 °C and the supernatant frozen at – 4 °C. The frozen supernatant was freeze-dried to give 2.5 g of lyophilized enzyme preparation as a pale yellow powder. For the test of enzymatic activity 250 mg of powder were dissolved in 20 mL of H_2O.

The assay of lipoxygenase activity was carried out according to Suda et al. [46] by mixing 200 μL of 10 mM sodium linoleate, 200 μL of 100 μM methylene blue, 1 mL of 50 mM borate buffer pH 9.0 and distilled water to a final volume of 2 mL. To this solution, kept under magnetic agitation, LOX-1 solution (2 mg/mL, 70 μL), soybean fresh extract (50 mg/mL, 20 μL) or solution of freeze-dried extract (100 μL, 12.5 mg/mL) was added and the absorbance at λ 660 nm was monitored over 10 min.

2.4. Biocatalyzed synthesis of 15S-hydroxy-(5Z,8Z,11Z,13E,17Z)-eicosapentaenoic acid, **1**

To a solution of EPA (100 mg, 0.33 mmol) in 50 mM borate buffer at pH 9.0 (20 mL) soybean lyophilized enzyme preparation (200 mg) and sodium borohydride (25 mg, 0.66 mmol) were added. The reaction mixture was stirred at room temperature under a flow of air (100 mL/min) until complete disappearance of the substrate (1 h) and then taken to pH 3 by addition of dil. HCl. The resulting suspension was filtered through a Celite pad and the clear filtrate was discarded. The Celite pad was suspended in 60 mL of EtOAc and stirred for 15 min. After filtration, the organic phase was dried with Na_2SO_4 and the solvent evaporated under vacuum to give an oily residue (130 mg) which was purified by column chromatography (Si gel 3.0 g, from *n*-hexane: HCOOH 99:1 to *n*-hexane:AcOEt:HCOOH 93:6:1, 150 mL) to give pure **1** (74 mg, 70% yield). $[\alpha]_D$ = + 4.4 (*c* 0.88, MeOH), lit. [47] $[\alpha]_D$ = + 4.9 (*c* 0.11, MeOH). ^{1}H NMR: *δ* 0.97 (t, 3H, *J* = 7.6, H-20), 1.71 (br q, 2H, H-3), 2.09 (m, 2H, H-19), 2.14 (br q, 2H, H-4), 2.38–2.35 (m, 4H, H-2 and H-16), 2.82 (m, H-7), 2.98 (m, 2H, H-10), 4.28 (br q, 1H, H-15), 5.32–5.48 (m, 6H, H-5, H-6, H-8, H-9, H-11, H-17), 5.57 (m, 1H, H-18), 5.73 (dd, 1H, *J* = 5.6 and 15.2, H-14), 6.00 (t, 1H, *J* = 10.8, H-12), 6.60 (dd, 1H, *J* = 11.2 and 15.2, H-13). ^{13}C NMR: *δ* 14.2 (C-20), 20.7 (C-19), 24.4 (C-3), 25.7 (C-7), 26.2 (C-10), 26.4 (C-4), 33.0 (C-2), 35.2 (C-16), 72.0 (C-15), 123.5 (CH =), 125.4 (C-13), 127.4 (CH =), 127.8 (C-12), 128.6 (CH =), 128.8 (CH =), 128.9 (CH =), 130.3 (CH =), 135.2 (C-14), 135.4 (C-18), 178.1 (C-1). HR-ESI-MS: 317.21230 [M – H]$^-$; theor. for $C_{20}H_{29}O_3^-$ 317.21112

2.5. Synthesis of compound **3**

According to a reported procedure [48] compound **1** (30 mg, 0.094 mmol) was converted into the corresponding methyl ester by reaction with dimethylcarbonate (0.5 mL) in the presence of Novozym 435 lipase (30 mg). The reaction mixture was stirred (280 rpm) in a shaker at 55 °C until complete conversion of the substrate was reached (5 h), then quenched by filtration of the enzyme. The solution was taken to dryness under vacuum and the residue was purified by column chromatography (Si gel, from *n*-hexane to *n*-hexane:EtOAc to 9:1) to afford ester **2** (29 mg, 0.09 mmol, 96% yield) as a colorless oil. Compound **2** (10 mg, 0.031 mmol) was dissolved in CH_2Cl_2 (0.8 mL) and to this solution *N,N'*-dicyclohexylcarbodiimmide (DCC, 0.3 mmol), dimethylaminopyridine (DMAP, 0.15 mmol) and (*R*)-(–)-methoxyphenylacetic acid (MPA, 0.15 mmol) were added. The mixture was left at room temperature under stirring for 18 h. After evaporation of the solvent, the residue was purified by column chromatography (Si gel 1.5 g, from *n*-hexane to *n*-hexane:EtOAc 9:1 v/v, 80 mL) to give 12 mg (0.025 mmol, 82% yield) of diester **3** as a colorless oil. ^{1}H NMR: *δ* 0.89 (t, 3H, *J* = 7.6, H-20), 1.72 (m, 2H, H-3), 1.92 (m, 2H, H-19) 2.12 (m, 2H, H-4), 2.25–2.35 (m, 4H, H-2 and H-16), 2.81 (br t, H-7), 2.92 (br t, 2H, H-10), 3.42 (s, 3H, -OMe), 3.67 (s, 3H, -COOMe), 4.76 (s, 1H, -*CH*OMe), 5.05 (m, 1H, H-17) 5.35–5.46 (m, 7H, H-5, H-6, H-8, H-9, H-11, H-15, H-18), 5.62 (dd, 1H, *J* = 7.2 and 15.2, H-14), 5.95 (t, 1H, *J* = 10.8, H-12), 6.52 (dd, 1H, *J* = 11.2 and 15.2, H-13), 7.35 (m, 3H, Ph), 7.44 (br d, 2H, Ph). ^{13}C NMR: *δ* 14.0 (C-20), 20.6 (C-19), 24.8 (C-3), 25.6 (C-7), 26.1 (C-10), 26.5 (C-4), 32.1 (C-2), 33.4 (C-16), 51.5 (–$COOCH_3$), 57.3 (–OCH_3), 75.0 (C-15), 82.7 (-*CH*OMe), 122.4 (C-17), 127.2 (2 × Ar-C), 127.4 (CH =), 127.7 (C-12), 128.0 (C-13), 128.5 (2 × Ar-C), 128.6 (CH =), 128.8 (CH =), 129.1 (CH =), 130.5 (C-14), 131.5 (CH =), 134.7 (C-14), 136.3 (C-1 Ar), 170.0 (MPA-CO), 174.0 (C-1).

2.6. Test for peroxygenase activity

Commercial whole seeds (60 g) of air-dried oat (*Avena sativa*) from organic crops were ground by an electric coffee mill and the obtained

flour was defatted by suspension in diethyl ether (150 mL) for 10 min followed by centrifugation at 2930 *g* for 10 min. The surnatant was discarded and the procedure repeated on the residue another two times. The final residue was dried at room temperature overnight to give 56 g of defatted flour.

For test reactions on flour, the defatted oat flour (2 g) was suspended in 50 mM potassium phosphate buffer pH 7.5 (7 mL) and the mixture used as a whole. For test reactions on suspensions, the defatted oat flour (2 g) was suspended in the phosphate buffer, mixed under magnetic stirrer for 5 min and centrifuged at 2930 *g* for 2 min. The bottom fraction was discarded and the suspension was used for the epoxidation reaction by adding the reagents.

Freeze-dried preparation of peroxygenase was obtained as follows. Defatted oat flour (110 g) was suspended in water (200 mL) and mixed under magnetic stirrer for 5 min. The mixture was centrifuged at 2930 *g* for 2 min and the suspension was collected. The procedure was repeated again on the bottom fraction of the centrifugation and the pooled suspension fractions were freeze-dried to give 15.2 g of a white light powder.

In the test for peroxygenase activity, defatted oat flour (2 g) or the suitable enzyme preparation deriving from 2 g of flour was suspended in 7 mL of 50 mM potassium phosphate buffer at pH 7.5. To this suspension methyl oleate (13 μL, 11.4 mg, 38 μmol) and *t*-BuOOH (70 wt% in H_2O, 13 μL, 8.5 mg, 95 μmol) were added and the reaction mixture was maintained under vigorous stirring at 25 °C. The reaction progress was monitored by GC analysis of aliquots (0.4 mL) of the reaction taken at regular intervals and extracted with MeOH:Et_2O 1:9 v/v (0.4 mL); 3 μL of the dried organic solution were injected for the GC analyses.

2.7. Biocatalyzed synthesis of (17R,18S)-epoxy-(5Z,8Z,11Z,14Z)-eicosatetraenoic acid, 4

To a suspension of freeze-dried oat extract (550 mg) in 50 mM phosphate buffer at pH 7.5 (28 mL) containing EPA (100 mg, 0.33 mmol), *t*-BuOOH (70 wt% in H_2O) was added in three equal aliquots at time 0, 15 and 40 min (total volume 40 μL, 0.29 mmol). The reaction mixture was maintained for 2 h at 25 °C under vigorous magnetic stirring and monitored by TLC analysis (Si gel, *n*-hexane: EtOAc 7:3 v/v containing 0.8% of HCOOH). The reaction was quenched by addition of MeOH (0.5 mL) and the mixture was then extracted with Et_2O (3 × 15 mL). The collected organic layers were dried over Na_2SO_4 and taken to dryness under vacuum. The residue was purified by column chromatography on LiChroprep Si 60 (6 g) by eluting with *n*-hexane:EtOAc 9:1 v:v containing 0.8% of HCOOH (200 mL). Unreacted EPA (20 mg, 0.066 mmol, 20% yield) was eluted as the first fraction followed by compound **4** which was obtained as clear oil (46 mg, 0.14 mmol, 44% yield) with 65% *ee* (from analysis of the corresponding methyl ester), $[\alpha]_D^{25}$ = + 3.5 (*c* = 0.84, CH_3OH); lit. [14] $[\alpha]_D^{25}$ = +7.0 (*c* = 1.0, $CHCl_3$). ^{1}H NMR: δ 1.06 (t, 3H, *J* = 7.6, H-20), 1.51–1.66 (m, 2H, H-19), 1.72 (q, 2H, *J* = 7.6, H-3), 2.14 (q, 2H, *J* = 7.6, H-4), 2.19–2.26 (m, 1H, H-16a), 2.36 (t, 3H, *J* = 7.6, H-2), 2.41–2.48 (m, 1H, H-16b), 2.80–2.85 (m, 6H, H-7, H-10, H-13), 2.91–2.96 (m, 1H, H-18), 2.97–3.02 (m, 1H, H-17), 5.33–5.44 (m, 6H, H-5, H-6, H-8, H-9, H-11, H-12), 5.44–5.57 (m, 2H, H-14 and H-15). ^{13}C NMR: δ 10.6 (C-20), 21.0 (C-19), 24.5 (C-3), 25.6 (C-7, C-10), 25.8 (C-13), 26.1 (C-16), 26.4 (C-4), 33.2 (C-2), 56.7 (C-17), 58.6 (C-18), 124.3 (C-15), 127.8 (CH═), 128.0 (CH═), 128.2 (CH═), 128.4 (CH═), 128.8 (C-5), 128.9 (CH═), 130.5 (C-14), 178.6 (C-1). HR-ESI-MS: 317.21231 $[M - H]^-$; theor. for $C_{20}H_{29}O_3^-$ 317.21112. Fractions containing mixtures of monoepoxides (15.5 mg, 0.049 mmol) and isomeric diepoxides (11 mg, 0.033 mmol) accounted for 15% and 10% yield, respectively.

2.8. Stereochemical analysis of compound 4

Compound **4** (20 mg, 0.062 mmol) was dissolved in dimethylcarbonate (0.5 mL) and Novozym 435 lipase (20 mg) was added. The reaction mixture was stirred in a shaker at 55 °C until complete conversion of the substrate was reached (2 h), then quenched by filtration of the enzyme. The solution was taken to dryness under vacuum and the residue was purified by column chromatography using LiChroprep Si 60 (0.8 g) and eluting with *n*-hexane:EtOAc 95:5 (30 mL) to afford ester **5** (19 mg, 0.042 mmol, 92% yield) as a colorless oil, $[\alpha]_D^{25}$ = +3.5 (*c* = 0.17, CH_3OH); lit. [14] $[\alpha]_D^{25}$ = +5.0 (c = 1.0, CH_3OH). ^{1}H NMR: δ 1.05 (t, 3H, *J* = 7.6, H-20), 1.52–1.65 (m, 2H, H-19), 1.72 (q, 2H, *J* = 7.2, H-3), 2.11 (q, 2H, *J* = 7.2, H-4), 2.20–2.27 (m, 1H, H-16a), 2.33 (t, 3H, *J* = 7.2, H-2), 2.38–2.43 (m, 1H, H-16b), 2.80–2.85 (m, 6H, H-7, H-10, H-13), 2.88–2.92 (m, 1H, H-18), 2.94–2.98 (m, 1H, H-17), 3.67 (–OCH_3), 5.37–5.39 (m, 6H, H-5, H-6, H-8, H-9, H-11, H-12), 5.47–5.51 (m, 2H, H-14 and H-15). ^{13}C NMR: δ 10.6 (C-20), 21.0 (C-19), 24.8 (C-3), 25.6 (C-7, C-10), 25.8 (C-13), 26.2 (C-16), 26.5 (C-4), 33.4 (C-2), 51.4 (–OCH_3), 56.5 (C-17), 58.3 (C-18), 124.5 (C14, C15), 127.8 (CH═), 128.0 (CH═), 128.3 (CH═), 128.4 (CH═), 128.8 (C-5), 130.4 (C14/C15), 174.0 (C-1). HR-ESI-MS: 355.22426 $[M + Na]^+$; theor. for $C_{21}H_{32}O_3Na^+$ 355.22437.

The enantiomeric excess (65% *ee*) was measured on a Lux Amylose-1 column (Phenomenex) eluting with a 0.7 mL/min flow of *n*-hexane/2-PrOH 99:1 at 23 °C; t_R/min: 12.69 [minor, (17*S*,18*R*)-enantiomer] and 24.16 [major, (17*R*,18*S*)-enantiomer].

3. Results and discussion

3.1. Lipoxygenase activity in soybean flour extracts

At the onset of our study, the lipoxygenase activity in soybean flour extracts was tested on linoleic acid by using a simple colorimetric test based on the bleaching of methylene blue. In the presence of lipoxygenase, indeed, linoleic acid is converted into the corresponding hydroperoxide which reduces methylene blue to its colourless leucoform and the enzyme activity can be monitored by following the decrease over time in the UV absorbance (λ 660 nm) of the reaction mixture [46].

By this way, it was observed that simple stirring of defatted soybean flour with water was sufficient to extract lipoxygenase in solution, with an optimal extraction time of 30 min, and the activity of the extracted enzyme (3000 U/mg of soybean flour) was determined by comparison with that of commercial lipoxygenase LOX-1 (Fig. 1).

The preliminary defatting of soybean flour did not affect the enzymatic activity, but a significant loss of activity in the extract (as well as in the solution of LOX-1) was detected as early as 2 h from extraction

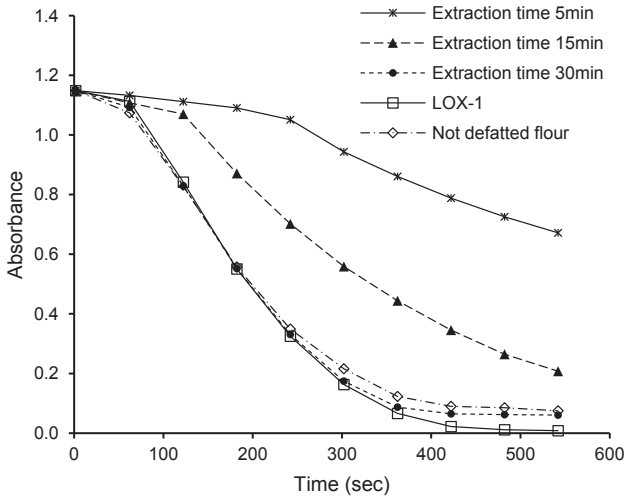


Fig. 1. Lipoxygenase activity in water extracts of defatted soybean flour in comparison with commercial LOX-1 and extract from non defatted soybean flour.

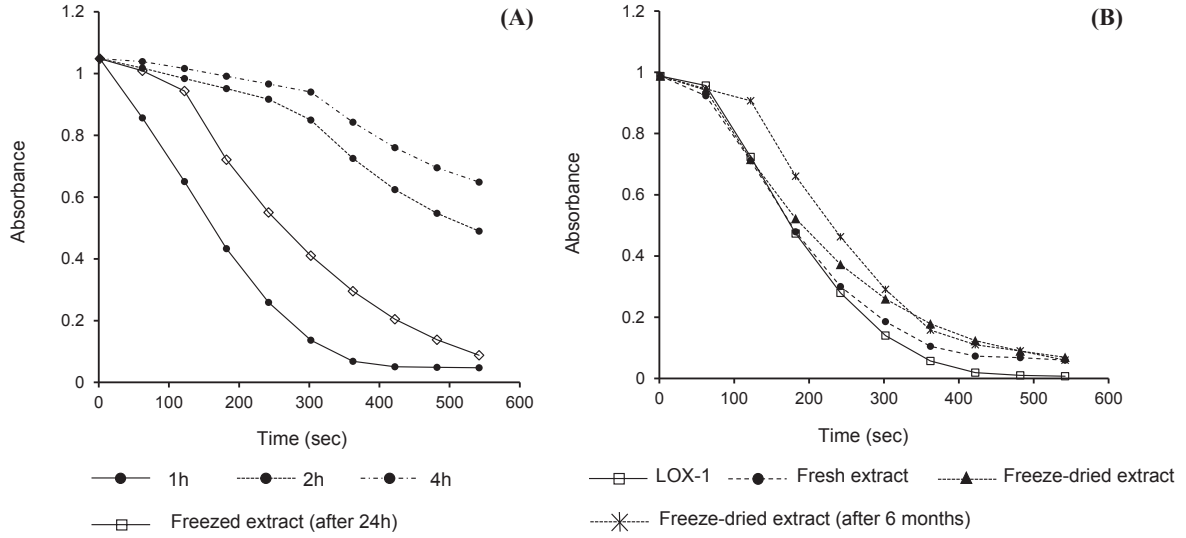


Fig. 2. (A) Lipoxygenase activity in soybean flour extracts at different times after extraction; (B) lipoxygenase activity in freeze-dried extracts (100 μL) compared with fresh extract (20 μL) and purified enzyme. (Also see the experimental section).

(Fig. 2A), so that the enzymatic conversion of less reactive substrates could pose some concerns. The freezing of the extract was not sufficient to maintain the enzymatic activity so that the extract should be freshly prepared for its use in biocatalyzed reactions.

In this context, we envisaged that the freeze-drying of soybean flour extract could be advantageous in giving the availability of active enzyme as if it was freshly extracted and the possibility to portionwise add the enzyme in case of slow-reacting substrates without volume increase. Furthermore, the reproducibility between different reactions could be ensured by using the same lot of lyophilized enzyme preparation.

Thus, soybean flour was stirred in water for 30 min and the solid separated by centrifugation; the solution was then freeze-dried and about 20% of the activity of the fresh extract was recovered in the obtained enzyme preparation, probably due to the aforementioned time-dependent deactivation, inherent in the enzyme, during the preparation and lyophilization of the sample. However, it is noteworthy that the recovered lipoxygenase activity was still present after 6 months of storage at –20 °C and the ultimate reaction rate was the same as the freshly freeze-dried enzyme preparation (Fig. 2B).

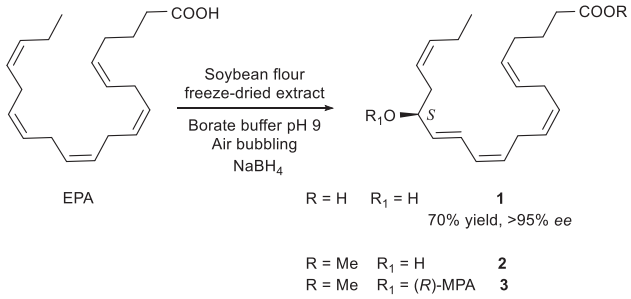
3.2. Biocatalyzed hydroperoxidation of EPA

Although the hydroperoxidation of fatty acids by soybean lipoxygenase has been widely investigated, an optimal experimental procedure is not still established and a variety of conditions have been adopted in terms of temperature, pressure of oxygen, substrate concentration and amount of enzyme.

In most cases the enzyme displays optimal activity and selectivity at pH 9.0 [31] giving an hydroperoxide which is subsequently chemically reduced to the corresponding alcohol.

In our experiments, we chose to dissolve the lyophilized preparation of lipoxygenase in 50 mM borate buffer at pH 9.0, and magnetically stir the reaction mixture at room temperature under a vigorous bubbling of air. In a first reaction, full conversion of 1.65 mM EPA substrate in the presence of 1.8 mg/ml of soybean freeze-dried extract was reached within 1 h and the subsequent chemical reduction of the crude reaction mixture with $NaBH_4$ gave the expected monohydroxy derivative **1** as almost exclusive product (Scheme 2).

Increasing the substrate concentration led to unreacted EPA even after 2 h and complete conversion of the substrate was achieved only after the addition of fresh enzyme. This behaviour has been reported for other PUFAs and attributed to inhibition/deactivation of lipoxygenase by substrate and/or product or other reactive oxygen species. In this



Scheme 2. Biocatalyzed synthesis of 15-OH EPA with soybean flour extract.

context, the simultaneous presence of a water-soluble triphenylphosphine as reducing agent during the enzymatic oxidation has been proposed to overcome the problem [33].

Among the different reducing agents we have tested, $NaBH_4$ in a 2:1 M ratio with respect to the substrate was the best and allowed the concentration of EPA to be increased up to 24 mM maintaining a full conversion within 1 h. When a preparative reaction was carried out we faced with extensive loss in the recovered product, due to the formation of a gelatinous emulsion during the extraction of the acidified reaction mixture with an organic solvent so that the reported work-up protocol was modified. In our optimized conditions, HCl was added to the reaction mixture up to pH 3 and the resulting cloudy suspension was filtered over a Celite pad in a sintered funnel; the clear filtrate was then discarded and the Celite washed with EtOAc to give an organic fraction containing the alcohol **1**. In the ^{1}H NMR spectra of **1**, in addition to the diagnostic broad quartet of the –C*H*OH proton at 4.3 ppm, three well-resolved resonances in the region of ethylenic protons were observed and associated with the two conjugated double bonds, in which two hydrogens are in a *trans* configuration as deduced from their large coupling constant (J = 15.2 Hz). The presence of just two methylene groups located between two double bonds, recognizable for their peculiar chemical shift around 2.8–3.0 ppm is compatible with the functionalization of C8-C9 or C14-C15 double bond and the discrimination between the two possibilities was obtained by 2D experiments. Indeed, $^3J_{HC}$ correlation of the C20 triplet with an ethylenic carbon in the 2D-HMBC spectrum allowed to unequivocally assign the C-18 carbon resonance, from which the H-16 proton was identified and found scalarly coupled (2D-COSY) with the −C*H*OH proton.

The structure of **1** was then established as 15-hydroxy-(5Z,8Z,11Z,13E,17Z)-eicosapentaenoic acid and the *S*-configuration of the molecule was assigned by comparison of its optical rotation with literature data [47], so confirming the stereoselectivity of soybean lipoxygenase. Further support came from the ^{1}H NMR spectrum of the diester **3**, obtained by reaction of the ethyl ester **2** with (*R*)-α-methoxyphenylacetic acid (MPA), that was in agreement with the (*R*)-MPA-15*S*-diastereoisomer [46]. The resonances reported for the (*R*)-MPA-15*R*-diastereoisomer,[1] were not visible in the spectrum of **3** (see supporting information) thus the enantiomerically pure nature of **1** was established. For alcohol **1**, which has been recently isolated from some microalgae [47] and marine diatoms [49,50], interesting antibacterial [51] and anti-inflammatory [47] activities have been reported.

3.3. Peroxygenase activity in oat flour extracts

In oat seeds the peroxygenase is localized in the microsomial fraction, which can be separated from the other cellular components by ultracentrifugation to give an enriched enzyme preparation [39,41,42]. As an alternative, the whole oat seed flour has been directly used for some biocatalyzed transformations [43]. A procedure for the immobilization of the enzyme activity on a protein-binding synthetic membrane has been also developed and the resultant preparation was tested either in aqueous medium or organic solvents [52,53].

Encouraged by the results obtained with the freeze-dried extract of soybean flour, we were interested the possibility to apply the same procedure also to oat flour extracts. Since the peroxygenase from oat is not available in purified form, the different enzyme preparations were compared for their activity in a standard reaction of methyl oleate in the presence of *t*-butyl hydroperoxide (*t*-BuOOH) as oxidant, by monitoring the reaction course by GC-MS analysis. When H_2O_2 was tested as alternative oxidant, the enzymatic activity was fully inhibited and only the unreacted substrate was detected.

In the first reactions the substrate was added to the whole slurry obtained by homogenization of the oat flour in phosphate buffer at pH 7.5 [43] and preliminary defatting of the flour was found to be essential for the peroxygenase activity (Table 1, entries 1 and 2), which was maintained also after 2 h from extraction (entry 3).

Centrifugation of the slurry at 2930 *g* allowed to discard a solid fraction and recover the enzyme activity in the microsome-containing surnatant, which appeared as a white milky suspension. When methyl oleate was added to this suspension it was fully converted into the corresponding epoxide in 1 h and the increase in the reaction rate could be related with a better contact between the enzyme and reactants, favoured by the lower density of reaction mixture compared with that containing the whole homogenate (Table 1, compare entries 2 and 4).

Centrifugation of the slurry at 11,000 *g* gave a clear solution with about halved activity with respect to the above cited suspension (Table 1, compare entries 4 and 5). Homogenization of the oat flour with water instead the phosphate buffer and subsequent centrifugation gave a suspension that was freeze-dried to afford a white light powder (5.5% weight with respect to flour) with slightly lower activity with respect to the parent enzyme preparation (Table 1, compare entries 6 and 4). The activity of the freeze-dried suspension was monitored up to 6 months (storage at –20 °C) without giving significant differences, thus ensuring reproducibility within the same batch of enzyme preparation.

Differences in the enzymatic activity could be expected depending on the biological variability of the oat source, the efficiency of centrifugation and the yield of lyophilized powder. Our enzyme preparation converted 0.32 μmol of methyl oleate/mg of lyophilized enzyme preparation in 1 h.

3.4. Biocatalyzed epoxidation of EPA

The epoxidation of PUFAs by peroxygenase from oat has been previously applied only to linoleic and linolenic acids (or their methyl esters), that were converted in mixtures of the corresponding monoepoxides and diepoxides [42]. Stereoselective epoxidation of PUFAs with higher degree of unsaturation by different human cytochromes has been reported [54–56], but these studies are mostly directed to understanding the *in vivo* metabolism of PUFAs and other oxygenases have not been investigated up to date.

The lack of data on a biocatalytic route to EPA-derived epoxides in preparative scale prompted us to test our lyophilized extract from oat flour as catalyst for the epoxidation reaction. The enzyme preparation was then suspended in phosphate buffer (pH 7.5) and EPA was left to react in the presence of *t*-BuOOH as oxidant (*t*-BuOOH:EPA 5:1 M ratio, three equal aliquots) at room temperature. The substrate was quickly converted into a mixture of mono- and poly- epoxide derivatives, so evidencing that oat peroxygenase is not selective toward the monofunctionalization of EPA and partially epoxidized derivatives of EPA are also good substrates for the enzyme. However, the time-course monitoring of the enzymatic reaction, in comparison with the chemical epoxidation of EPA in the presence of *m*-chloroperbenzoic acid, revealed that one monoepoxide among the five possible ones was mainly formed as transient product in the peroxygenase-catalyzed epoxidation. Thus, we focused our attention on the possibility to maximize the formation of a single monoepoxide derivative of EPA and a biocatalyzed reaction was carried out in the presence of 0.9 eqv. of *t*-BuOOH. In these conditions, 80% conversion of the substrate was reached in 2 h and epoxide **4** was obtained in 44% isolated yield and its structure was established by NMR-analysis (Scheme 3).

In the ^{1}H NMR spectrum, the signals in the region 5.3–5.6 ppm accounted for four ethylenic bonds and the two methinic protons on the oxirane ring were identified by their C—H correlation in the 2D-HSQC spectrum with signals at 56.7 and 58.6 ppm. Diagnostic correlation between the oxirane carbon at 58.6 and methyl protons on C-20 was observed in the 2D-HMBC spectrum, so that structure of 17,18-epoxy-(5Z,8Z,11Z,14Z)-eicosatetraenoic acid was unequivocally assigned to compound **4**. Compound **4** was isolated in 65% enantiomeric excess, as determined by chiral HPLC analysis of the corresponding methyl ester **5**, and the 17*R*,18*S* absolute configuration was assigned by comparison of optical rotation of **5** with data reported in literature [14]. However, we found an opposite elution order of the two enantiomers of **5** on the Amylose-1 (Phenomenex) HPLC column with respect the reported data for the same chiral stationary phase [14]. When our sample was analysed on a Chiralcel OB column the 17*R*,18*S*-enantiomer was eluted as the first peak, in agreement with previous studies [54], so confirming the assigned configuration of **5** and giving evidence that the elution order of enantiomers is opposite on Chiralcel OB and Amylose-1 columns.

Although epoxidation reactions catalysed by oat peroxygenase have been reported for a variety of ethylenic substrates, no information has been given regarding the stereochemistry and the optical purity of the obtained products. Thus, our data provide the first report of the stereochemical selectivity of oat peroxygenase.

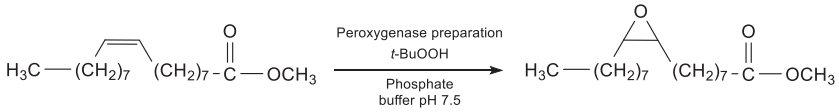
Positive effects in decreasing cell proliferation have been reported for compound **4**, that has been shown to be the only active EPA-derived monoepoxide compared to the other regioisomers [57], and its ability to regulate the contraction of cardiomyocytes [58] makes it attractive as antiarrythmic drug.

4. Conclusions

Simple and effective bioprocesses for the stereo- and regioselective preparation of two oxygenated derivatives of EPA were developed by

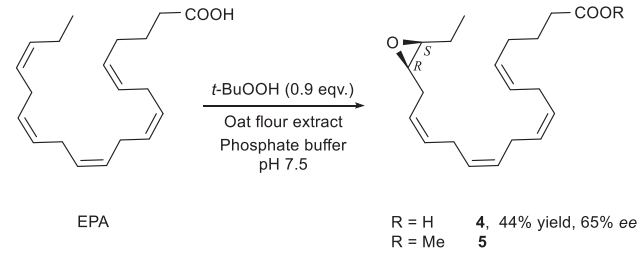
[1] For the (*S*)-MPA-15*S*-diastereoisomer (which is magnetically equivalent to (*R*)-MPA-15*R*-diastereoisomer) the following resonances (selected resonances) were reported: δ 0.95 (H-20), 2.76 (H-10), 5.25 (H-17), 5.51 (H-14), 5.85 (H-12), 6.28 (H-13).

Table 1
Activity of different peroxygenase preparation from oat flour[a].



Entry	Enzyme preparation	% Epoxide (0.5 h)[b]	% Epoxide (1 h)[b]
1	Slurry[c]	32	50
2	Slurry	43	82
3	Slurry[d]	44	75
4	Microsomes suspension	80	99
5	Microsomes suspension[e]	42	65
6	Freeze-dried suspension	74	92
7	Freeze-dried suspension[d]	71	88
8	Freeze-dried suspension[f]	72	88

[a] Experimental conditions: defatted oat flour (2 g) or enzyme preparation from 2 g of defatted flour; 50 mM phosphate buffer (7 mL), methyl oleate (0.013 mL, 0.038 mmol), 70% v/v of TBHP (0.013 mL, 0.1 mmol), 25 °C.
[b] By GC analysis of the reaction mixture.
[c] Flour was not defatted.
[d] Reagents were added after 2 h of stirring in phosphate buffer.
[e] From centrifugation at 11000 g.
[f] After 6 months from freeze-drying



Scheme 3. Biocatalyzed synthesis of 17,18-epoxy-EPA with oat flour extract.

exploiting the lipoxygenase and peroxygenase content of soybean flour and oat flour, respectively. The enzymatic activity was recovered from these flours in the aqueous extracts, which could be used as such in place of the purified enzymes. The freeze-drying of the aqueous extracts gave enzyme preparations stable up to 6 months and allowed reproducibility of the reactions and simplification of the workup. Further studies are in progress to extend the procedure to polyunsaturated fatty acids other than EPA, also targeting at the preparation of specific derivatives with relevant biological activities.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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