

## Article

# Synthesis of Linoleic Acid 13-Hydroperoxides from Safflower Oil Utilizing Lipoyxygenase in a Coupled Enzyme System with In-Situ Oxygen Generation

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**Abstract:** Linoleic acid hydroperoxides are versatile intermediates for the production of green note aroma compounds and bifunctional  $\omega$ -oxo-acids. An enzyme cascade consisting of lipoyxygenase, lipase and catalase was developed for one-pot synthesis of 13-hydroperoxyoctadecadienoic acid starting from safflower oil. Reaction conditions were optimized for hydroperoxidation using lipoyxygenase 1 from *Glycine max* (LOX-1) in a solvent-free system. The addition of green surfactant Triton CG-110 improved the reaction more than two-fold and yields of >50% were obtained at linoleic acid concentrations up to 100 mM. To combine hydroperoxidation and oil hydrolysis, 12 lipases were screened for safflower oil hydrolysis under the reaction conditions optimized for LOX-1. Lipases from *Candida rugosa* and *Pseudomonas fluorescens* were able to hydrolyze safflower oil to >75% within 5 h at a pH of 8.0. In contrast to *C. rugosa* lipase, the enzyme from *P. fluorescens* did not exhibit a lag phase. Combination of *P. fluorescens* lipase and LOX-1 worked well upon LOX-1 dosage and a synergistic effect was observed leading to >80% of hydroperoxides. Catalase from *Micrococcus lysodeikticus* was used for in-situ oxygen production with continuous  $H_2O_2$  dosage in the LOX-1/lipase reaction system. Foam generation was significantly reduced in the 3-enzyme cascade in comparison to the aerated reaction system. Safflower oil concentration was increased up to 300 mM linoleic acid equivalent and 13-hydroperoxides could be produced in a yield of 70 g/L and a regioselectivity of 90% within 7 h.

**Keywords:** lipoyxygenase; lipase; catalase; LOX; enzyme cascade; hydroperoxides; in-situ oxygen generation; linoleic acid; HPODE



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## 1. Introduction

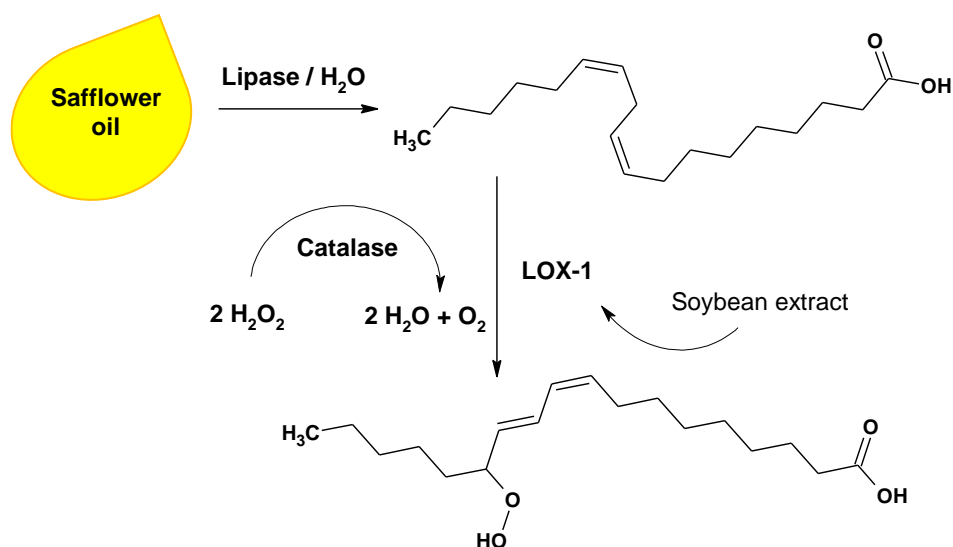
Lipoyxygenases (LOX, EC. 1.13.11.X) are widely distributed in eukaryotes and were more recently identified in bacteria as well [1]. They belong to the group of non-heme dioxygenases and catalyze the regio- and stereoselective hydroperoxidation of polyunsaturated fatty acids containing a (1Z, 4Z)-pentadiene system [2,3]. The reaction starts with a proton abstraction at position 3 of the pentadiene moiety and is followed by the antarafacial addition of molecular oxygen either in position 1 or 5 to form the corresponding fatty acid hydroperoxides [4]. In plants, linoleic and linolenic acid are natural substrates of LOX and depending on the enzyme's specificity either the 9R, 9S or 13S-hydroperoxide is formed [5]. In the lipoyxygenase pathway, the hydroperoxides are further metabolized into phytooxylipins such as green leaf volatiles, jasmonates or traumatic acid, which are involved in signaling and plant defense [6–8].

Applications of LOX include bleaching of colored components, utilization as baking additive or the synthesis of oxygenated lipid derivatives [9–11]. The main biocatalytic application is the production of natural green note aroma compounds for the flavor and fragrance industry by combined action of LOX and hydroperoxide lyase for splitting of the hydroperoxides [8,12,13]. This Hock-type rearrangement may also be catalyzed

chemically leading to volatile C6-aldehydes and the corresponding C12- $\omega$ -oxoacids [14,15]. The  $\omega$ -oxoacids are an interesting class of products and were proposed as bifunctional monomer precursors for green polymer synthesis [16]. Another possible LOX application could be the synthesis of plant metabolites like 12-oxophytodienoic acid (12-OPDA) in an enzymatic cascade mimicking the LOX pathway [17]. However, the utilization of linoleic acid rich oils for polymer application via the hydroperoxidation and cleavage route needs an optimized and cost-efficient production of the hydroperoxides.

Plant LOX are found in several fruits and vegetables, and especially soybeans possess a high concentration of different LOX isozymes making the beans the best natural source for LOX isolation and application in biocatalysis [18]. 13-S-specific LOX-1 obtained from soybean extracts was successfully applied for the synthesis of 13-S-hydroperoxides in yields up to 72% from either linoleic acid (13-HPODE) [19,20] or linolenic acid (13-HPOTE) [21,22]. A drawback of the utilization of soybean extracts is the presence of several LOX isozymes with either 9S-, 13S- or mixed 9/13-regioselectivities [18]. As separation of the LOX isozymes is tedious, alkaline pH-conditions were used in all syntheses to favor the 13S-specific LOX-1 activity over that of the other isozymes. Besides regioselectivity, solubility of the fatty acid substrates and strong foaming due to molecular oxygen injection under alkaline conditions, which leads to soap formation, are major issues in high-yield production of hydroperoxides with soybean LOX-1. Several studies used solvent-buffer systems and solvent-surfactant-microemulsions to overcome solubility issues [19,23–25]; however, overall sustainability of the process decreases, and solvent removal needs an extra processing step.

In previous attempts the combination of lipases and LOX was approached to catalyze oil hydrolysis and consecutive hydroperoxidation of the liberated fatty acids [26,27]. Solvent-buffer mixtures were needed for sufficient fatty acid release by the lipase, and the substrate concentrations were too low to allow for a technical process. A recent approach used a self-assembled system of immobilized lipase and LOX to convert soybean oil to hydroperoxides [28]. While the HPODE yield was higher than in previous works reported, substrate concentrations still seem too low for technical applications. In this work, we present the development of an enzymatic cascade consisting of lipase, LOX and catalase for efficient synthesis of linoleic acid hydroperoxides starting from safflower oil (Scheme 1). Lipases were screened for oil hydrolysis and catalase was implemented for in situ oxygen production with continuous  $H_2O_2$ -addition to prevent excessive foaming. After optimization of the enzyme cascade, solvent-free synthesis of 13-HPODE was possible in high yield and good regioisomeric specificity. Thus, our process offers a green and sustainable access to hydroperoxide precursors suitable for cost-efficient green note synthesis and oxo-acid production for polymer applications.

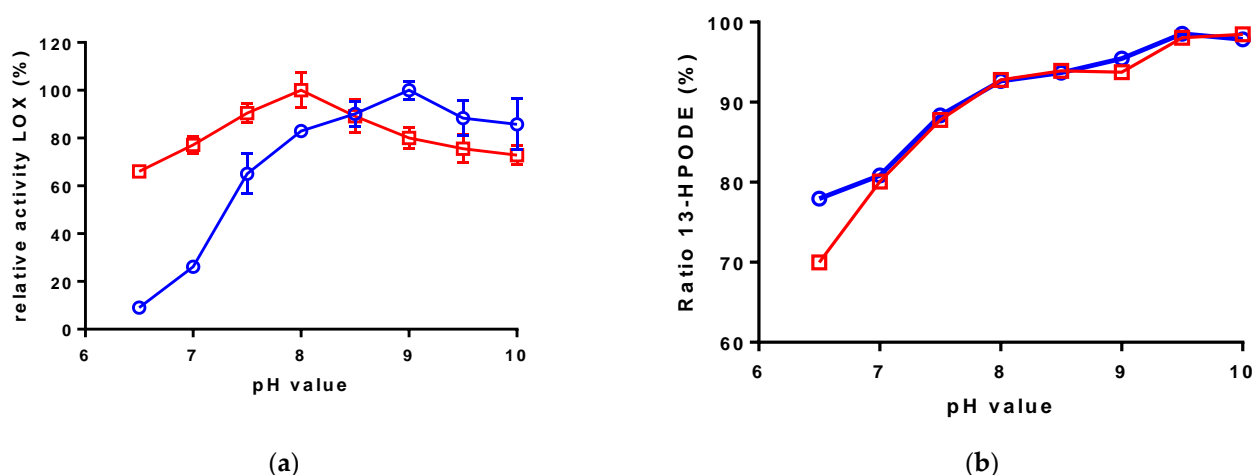


**Scheme 1.** Enzyme cascade comprising LOX, lipase and catalase.

## 2. Results & Discussion

### 2.1. LOX Preparation and Initial Optimization of LOX Reaction Conditions

A crude LOX preparation was prepared from soybeans by extraction of defatted meal and compared to commercially available LOX-1 preparation. The soybean extracts had activities of  $45 \pm 4$  U/mg ( $4500 \pm 395$  U/mL) with heptane and  $51 \pm 5.1$  U/mg ( $5100 \pm 506$  U/mL) with acetone as defatting agent; thus, the acetone extract was used for further studies. The commercially available LOX-1 exhibited pH optima of 9 regarding activity and  $>9$  regarding regioselectivity towards the 13-HPODE regioisomer (Figure 1). The soybean extract possessed a pH optimum of 8 and a more severe drop in regioselectivity was observed at neutral to acidic pH-values. Differences in activity and selectivity can be attributed to LOX isozymes 2 and 3 in the soybean preparations, which are known to possess 9- or mixed regioselectivity and higher activity at lower pH values [18]. With both preparations the pH-range from 8–10 was judged to be acceptable for the development of the enzyme cascade possessing both sufficient activity and regioselectivity.



**Figure 1.** (a) Comparison of specific activities and (b) of regioselectivities at a linoleic acid concentration of 1 mM in dependence of pH-values with  $\square$  = soy flour suspension and  $\circ$  = LOX-1 obtained from Sigma Aldrich (peak assignment of regioisomers is shown exemplarily in Figure S1).

The effect of substrate concentration and oxygen supply was examined in 50 mL scale using 20,000 U LOX /mmol of linoleic acid (Figure S2). In general, increasing the flow rate of  $O_2$  led to higher yields and a better isomeric ratio towards 13-HPODE. However, a sharp decrease in product formation was observed at substrate concentrations above 3 mM. While near quantitative yields were achieved at a linoleic acid concentration of 1 mM, only 12% were converted at 100 mM substrate concentration even at the highest oxygen addition rate of 100 mL/min. These results are in agreement with findings of other groups [21,29–31], pointing to an insufficient accessibility of the hydrophobic lipid substrate. Especially in small-scale experiments, hydroperoxidation efficacies were significantly lower than in larger scale experiments, in which a more intense mixing could be realized [21]. To overcome these limitations, homogenization strategies as well as solvent and surfactant addition were examined.

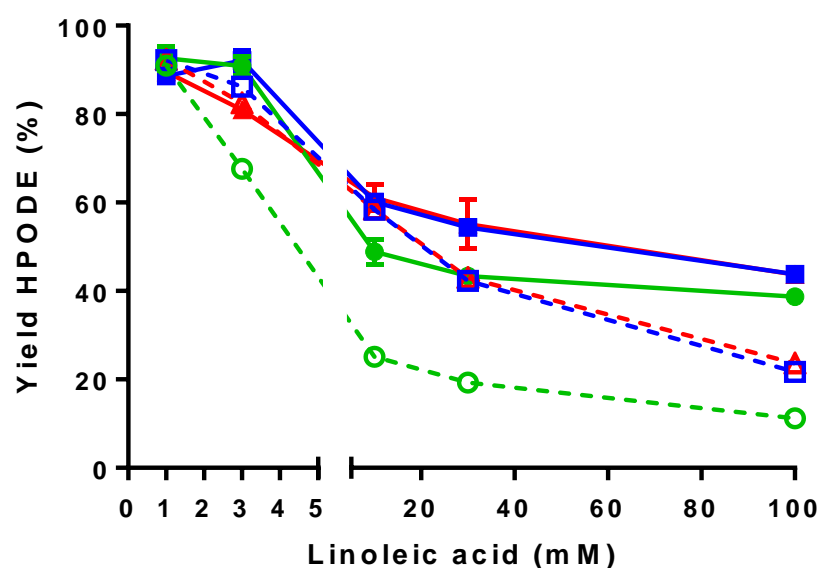
The application of homogenizer (Polytron PT 2500 E, Kinematica AG, Malters, Switzerland) and ultrasound (SonoPuls HD2200, Bandelin, Berlin, Germany) as pretreatment to improve emulsification led to higher peroxidation yields at linoleic acid concentration up to 30 mM. However, at higher substrate concentrations, the positive effect of this pretreatment vanished (Figure S3) and an increase in homogenization time to  $>30$  min did not lead to higher product yields either. Ten solvents of varying polarity were tested for their ability to improve substrate accessibility by increasing linoleic acid solubility (Figure S4). Though DMSO, heptane and 2-propanol led to a higher initial LOX activity, the overall substrate conversion after 2 h was lower with all solvents compared to solvent-free condi-

tions. Thus, neither mixing pretreatment nor solvent addition led to significantly improved hydroperoxide yields.

Additionally, 16 non-ionic surfactants of petrochemical and bio-based origin (Table 1) covering a range of HLB [32] values of 5–18 were evaluated at volumetric concentrations of 3 to 12% (*v/v* substrate). In all cases, a significantly higher product formation after 2 h was achieved with a moderate HLB-value dependence and an optimum HLB range between 8–13 (Figure S5). Triton CG-110, possessing an alkylpolyglycoside (APG) structure with C8/C10 fatty alcohols, showed the best performance with a 2.69-fold yield increase at the lowest concentration of 3% (*v/v* substrate) and was selected for further studies. APGs are fully biobased, readily biodegradable, possess low toxicity and are thus regarded as green surfactants [33], making them ideal additives for the development of a sustainable enzymatic process. The positive effect of Triton CG-110 was analyzed in the concentration range of 1–100 mM at different enzyme to substrate ratios. Above a linoleic acid concentration of 3 mM the surfactant effect became significant (Figure 2). Up to a LOX concentration of 100,000 U/mmol of substrate an increase in yield was obtained, whereas higher LOX concentrations improved the reaction only marginal. From these initial optimization studies, a solvent-free reaction system containing 100,000 U LOX-1/mmol substrate at a Triton CG-110 concentration of 3% (*v/v* substrate) was chosen.

**Table 1.** List of surfactants in order of their HLB value, black = non-biobased, blue = partially biobased and green = fully biobased surfactants; yield of HPODE in the presence of detergents (% relative to substrate volume) after 2 h of reaction time with 20,000 U LOX-1/mmol linoleic acid. Reference without detergent had a yield of 13.7%.

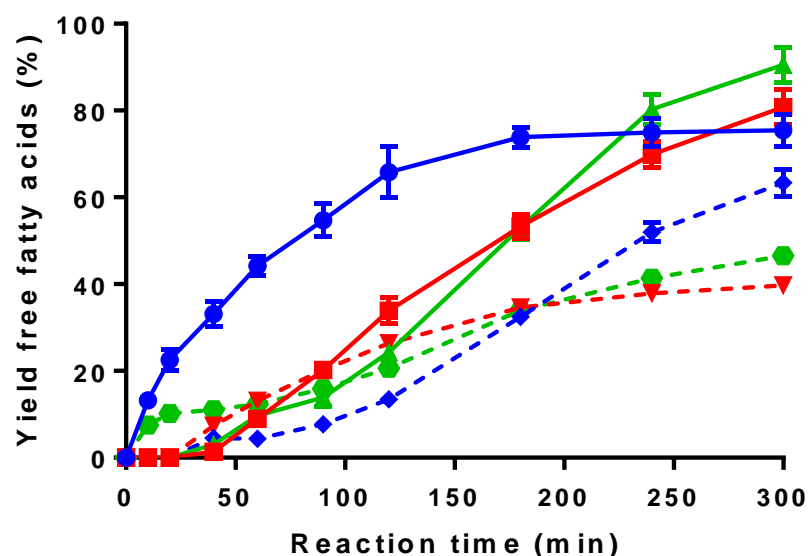
Surfactant Concentration:			3%	6%
Surfactant	HLB	Structure	Yield (%)	Yield (%)
Brij 93	4	Polyethylenglykol-oleylether	28.7	29.6
Span 80	4.3	Sorbitanmonooleat	25.5	24.7
Sophorolipid18:2	~6	Linoleic acid glucoside	26.2	28.4
Tergitol 15-S-3	8.0	Secondary Alcohol Ethoxylates	28.5	29.7
Ecosurf SA-9	11.1	Seed oil alcohols ethoxylated propoxylated	26.0	28.3
Brij O10	12.4	Polyoxyethylen-10-oleylether	27.1	27.8
Ecosurf EH-9	12.5	Ethyl hexanol ethoxylated propoxylated	33.3	29.9
Tergitol NP 9	12.9	Alkylphenol Ethoxylate (APE)	26.2	30.3
Triton CG-110	~13	Alkyl Polyglucoside	36.8	32.6
Tergitol 15-S-9	13.3	Secondary Alcohol Ethoxylates	27.6	28.3
Tergitol NP 10	13.3	Alkylphenol Ethoxylate (APE)	21.9	24.9
Triton X-100	13.4	Octyl phenol ethoxylate	28.5	27.8
Tween 80	15	Polyoxyethylene (20) sorbitan monooleate	23.9	23.7
Tergitol 15-S-20	15.6	Secondary Alcohol Ethoxylates	31.5	20.9
Tween 20	16.7	Polyoxyethylene (20) sorbitan monolaurate	16.5	30.8
Tergitol 15-S-40	18.0	Secondary Alcohol Ethoxylates	23.0	26.5



**Figure 2.** HPODE yield as a function of substrate concentration and enzyme–substrate ratio in the presence of 3% Triton-CG 110 (*v/v* substrate) with 20,000 U/mmol (○, ●), 100,000 U/mmol (□, ■) and 200,000 U/mmol (△, ▲) LOX, filled symbols: in presence of Triton-CG 110, open symbols and dotted line: without addition of surfactant.

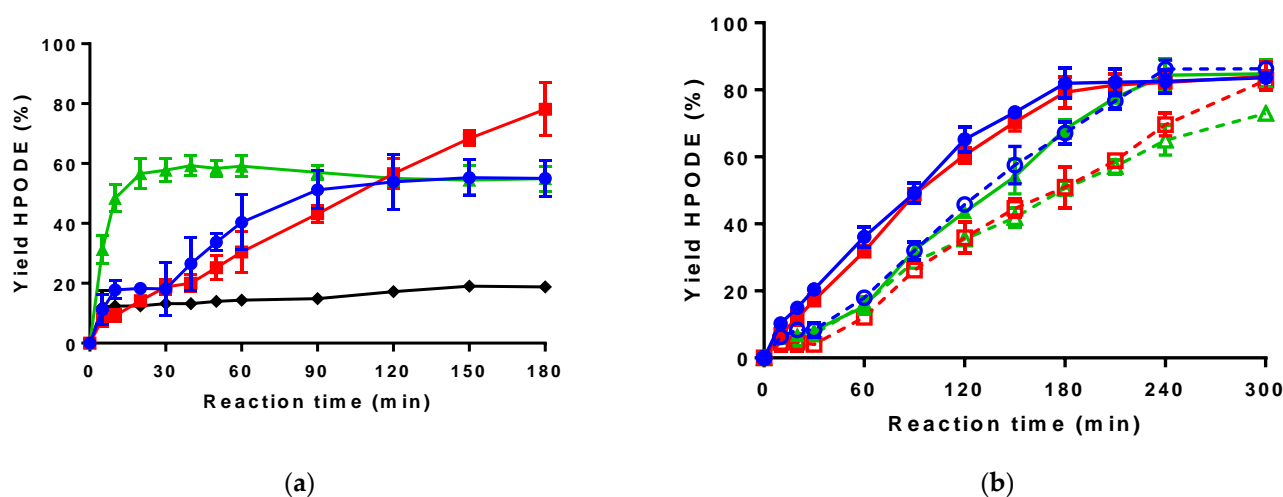
## 2.2. Lipase Selection and Evaluation of Bienzymatic Lipase-LOX-1 Reaction System

Lipases have been successfully employed in enzyme cascades in combination with a variety of oxidative enzymes or whole cells before. Typically, enzymes from *Candida rugosa* [34,35] or *Thermomyces lanuginosus* [36] were best suited for vegetable oil hydrolysis. A major issue of combining lipases with soybean LOX-1 is the alkaline pH optimum of the oxygenase. Gargouri et al. used high concentrations of lipase from either *Mucor javanicus* [26] or *Pseudomonas* sp. [27,37] in combination with LOX-1 under slightly alkaline conditions to liberate linoleic acid from trilinolein. We evaluated 12 commercially available lipases for their ability to hydrolyze linoleic-acid rich safflower oil. Under LOX-1 optimum conditions (50 mM borate buffer at pH 9 in the presence of Triton CG-110) none of the lipases could sufficiently hydrolyze the oil at a dosage of 5% (*w/v* substrate) (Figure S6A). *Pseudomonas fluorescens* lipase was the best enzyme with just 18% hydrolysis after 5 h under these conditions. Further tests showed that a lower pH value was essential to reach an acceptable degree of hydrolysis. As LOX-1 activity dropped fast with decreasing pH (Figure 1a), a pH value of 8.0 was chosen as compromise to maintain sufficient LOX and lipase activity. In 200 mM Tris-HCl buffer, up to 90% of hydrolysis was reached after 5 h with the *Candida rugosa* lipases, while lipases from *Mucor javanicus* and *Pseudomonas fluorescens* performed slightly weaker with 60–75% hydrolysis (Figure S6B). In time resolved hydrolysis experiments with safflower oil equivalent to 30 mM linoleic acid, it became apparent that most lipases exhibited a lag phase (Figure 3). Only the lipase from *P. fluorescens* showed a high initial reaction velocity and was thus favored over the *C. rugosa* lipases. Increasing the concentration of *P. fluorescens* lipase to 10% (*w/v* substrate) improved hydrolysis slightly, reaching 85% hydrolysis after 5 h.



**Figure 3.** Time dependent monitoring of safflower oil hydrolysis at pH 8 with 5% (*w/v* substrate) of ● = Amano lipase from *P. fluorescens*, ▲ = lipase CCL, ■ = lipase AY, ◆ = lipase M, ▼ = lipase F-AP 15 and ● = lipase PS.

Combining LOX-1 and *P. fluorescens* lipase did not lead to satisfactory results initially. When both enzymes were added at the start of the reaction, a hydroperoxide yield of 20% was obtained after 3 h according to UV 234 nm analysis and the reaction stopped after a few minutes (Figure 4a). A more detailed analysis revealed that the acid value was low corresponding to a degree of hydrolysis of just 12% in comparison to 80% of a control reaction with addition of lipase only (Figure S7, Table 2). The reason for the negative interdependence of the two enzymes is not fully clear. A reasonable explanation may be an interference of reactive hydroperoxidized glycerides with active site amino acids of the lipase resulting in deactivation of the enzyme.



**Figure 4.** (a) Hydroperoxidation starting from safflower oil with *P. fluorescens* lipase and LOX-1: ◆ = LOX-1 addition at start, ■ = LOX-1 dosage, ▲ and ● = comparative hydroperoxidation with linoleic acid and LOX-1 addition at start ▲/dosed ●; (b) Analysis of the hydroperoxidation yields based on free fatty acid content when using soy flour suspension and LOX-1 starting from safflower oil with *P. fluorescens* lipase. ●, ○ = HPODE yield at an initial safflower oil concentration equivalent to 30 mM linoleic acid with LOX-1/soy flour suspension. ■, □ = Yield of HPODE with LOX-1/soy flour suspension when adding safflower oil equivalent to 60 mM and 100 mM (▲, △) linoleic acid.



**Table 2.** Analysis of HPODE formation and degree of hydrolysis of comparative hydroperoxidation experiments shown in Figure 4a; Acid value was determined by titration of the lipid phase (Figure S6), HPODE ratio to residual linoleic acid was derived from GC analysis (Figure S8) and degree of hydrolysis was calculated from average molecular weight after hydroperoxidation and acid value.

Substrate	Linoleic Acid	Linoleic Acid	Safflower Oil	Safflower Oil	Safflower Oil
Enzyme Addition	LOX-1 Direct (▲)	LOX-1 Dosed (●)	LOX-1 Direct +Lipase (◆)	LOX-1 Dosed +Lipase (■)	Lipase Only (Control)
Acid value	176 ± 15	184 ± 9	23.5 ± 0.2	134 ± 9	160 ± 2
HPODE/linoleic acid ratio	67:33	64:36	61:39	84:16	-
Degree of hydrolysis	-	-	12 ± 0.1	72 ± 5	80 ± 1

It is known that LOX-1 acts preferably on the free fatty acids and glycerides are converted significantly slower [26]. A comparison of linoleic acid and monolinolein transformation in our reaction system confirmed this observation (Figure S8). Initial reaction velocity was approximately tenfold higher with linoleic acid as substrate. Therefore, to overcome lipase deactivation at least partially, a LOX-1 dosing strategy was applied. With less LOX-1 at the beginning of the reaction, oil hydrolysis should be favored over hydroperoxidation, and once linoleic acid is available in sufficient quantities the free fatty acid should be the preferred LOX-1 substrate. LOX dosages between 20,000 and 100,000 U/mmol substrate per hour were investigated (Figure S9) and at 20,000 U LOX/mmol lipase catalyzed hydrolysis and LOX hydroperoxidation showed similar conversion rates. Upon LOX dosing HPODE concentration increased to around 80% after 3 h, even exceeding the control reaction with LOX-1 dosage starting from linoleic acid (Figure 4a). To verify this surprising result analyzed by UV 234 nm, an additional GC-based quantification was established. After hydrogenation of the hydroperoxides with boron hydride, analysis of hydroxides and residual linoleic acid was possible in the presence of glycerides with a high-temperature GC-column (Figure S10). The hydroperoxide to linoleic acid ratio of the reaction with combined lipase hydrolysis and LOX-1 dosage was 84:16 in comparison to 64:36 starting from linoleic acid (Table 2) confirming the UV analyses. Glycerol released from safflower oil slightly enhanced initial LOX hydroperoxidation but did not influence the final hydroperoxide yield. The synergistic performance of lipase and LOX-1 may be explained by formation of partial glycerides, which improves emulsification and substrate accessibility for LOX-1 in combination with Triton CG-110. It should be mentioned that the degree of hydrolysis was 8% lower than in the control reaction without LOX-1 addition (Table 2) pointing to a partial lipase deactivation. Hence, a further optimization of LOX-1 dosage or application of lipase combinations may improve the overall hydrolysis rate.

The hydroperoxidation with LOX-1 dosage yielded >83% hydroperoxides within 5 h at a safflower concentration corresponding to up to 100 mM linoleic acid (Figure 4b). In combined lipase-LOX-1 systems previously reported [26,37], larger amounts of enzyme (up to 150% *w/w* of lipase and 25% *w/w* of LOX-1) had to be used and solvent was needed for lipase accessibility. The hydroperoxide yields at 100 mM linoleic acid concentration were approximately 4 times lower than in our reaction system. Largely, these higher yields can be attributed to the utilization of green surfactant Triton CG-110 and the implementation of a LOX-1 dosage strategy.

Comparative experiments with soy flour extract proceeded slower, but still > 83% hydroperoxides were produced within 5 h at starting concentrations up to 60 mM (Figure 4b, dotted lines). Only at an initial oil concentration of 100 mM linoleic acid equivalent the final hydroperoxide concentration was below 80%. Disadvantages of the soy flour extracts were the comparative low LOX activity (5100 U/mL) leading to a dilution upon biocatalyst dosage and significantly stronger foaming. Though addition of 0.2% silicone oil antifoam reduced foam formation to some extent, purified LOX-1 from soybean or recombinant LOX [38] would be the preferred biocatalysts for scale-up.

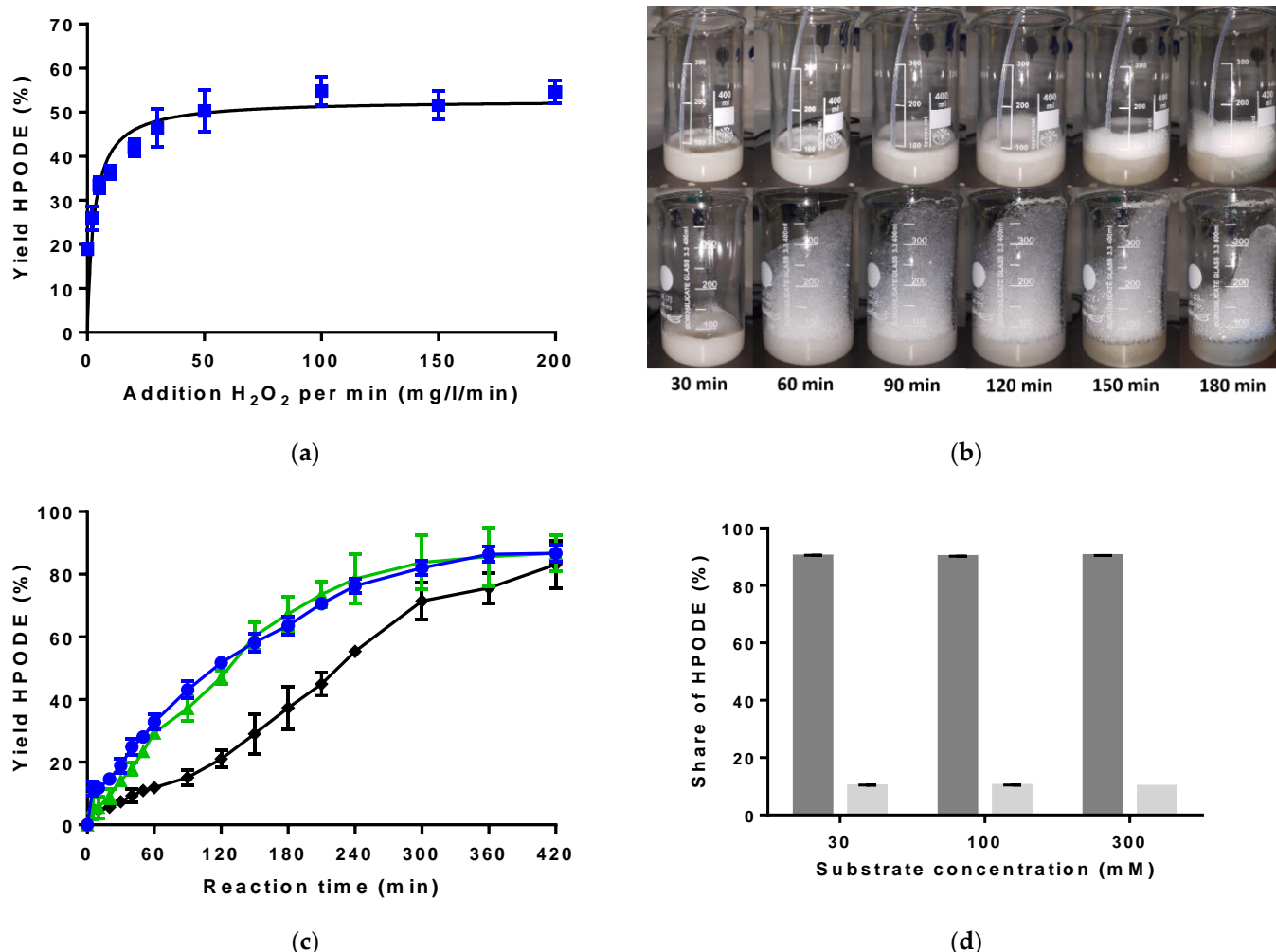
### 2.3. Implementation of an Enzyme Cascade with LOX-1, Lipase and Catalase

Application of catalase in biocatalytic oxidation reactions has been shown for  $\text{H}_2\text{O}_2$  generating oxidases, like e.g., pyranose 2-oxidase [39], amino acid and glucose oxidase [40] or galactose oxidase [41], which all use molecular oxygen as substrate. The catalase in these systems possess a dual use of keeping  $\text{H}_2\text{O}_2$  concentrations below a toxic level and generating dissolved  $\text{O}_2$  simultaneously. In our reaction system, a dual use for catalase was intended as well, though with a different focus: Besides generating dissolved or finely dispersed oxygen for the LOX-1 catalyzed hydroperoxidation, foam reduction was a major issue in the multiphase reaction system. In previous studies, we observed less foam generation when combining LOX and catalase for polyunsaturated fatty acid ester hydroperoxidation [42]. In our newly developed reaction system, addition of surfactant Triton CG-110 and intermediate formation of partial glycerides during safflower oil hydrolysis makes foam control essential for larger scale application. Catalase from *Micrococcus lysodeikticus*, a cost-efficient industrial enzyme with high enzymatic activity over a broad pH range was chosen for in-situ oxygen production. It is known that hydrogen peroxide is capable of denaturing proteins even at low concentrations [43]. The enzyme concentration was adapted to a value, which guaranteed the immediate hydrolysis of  $\text{H}_2\text{O}_2$  to prevent non-selective oxidation of the lipids and oxidative enzyme degradation. Under continuous  $\text{H}_2\text{O}_2$  dosage at least 1000 U/ $\mu\text{mol}$  hydrogen peroxide were needed for continuous substrate conversion over the course of reaction (Figure S11). To ensure full hydrogen peroxide conversion in the presence of lipoxygenase, catalase was added in 20-fold excess to the minimum amount needed.

The in-situ oxygen production was tested in combination with LOX, and peroxidation was monitored at different  $\text{H}_2\text{O}_2$  dosage rates. At 100 mg/mL/min  $\text{H}_2\text{O}_2$  the hydroperoxidation yield was comparable to the oxygen aerated system (Figure 5a), which typically yielded around 55–60% hydroperoxides (Figure 4a). In addition, foam generation was reduced significantly in comparison to the aerated system, when safflower oil was used as substrate in combination with lipase, LOX-1 and catalase (Figure 5b). Strong foaming was observed in the aerated system from 60 min onwards, which is probably caused by an increase of amphiphilic compounds comprising partial glycerides, saponified fatty acids and the hydroperoxides themselves in addition to Triton CG-110. In contrast, only a moderate foam formation was observed over the time course of 3 h in the catalase-based 3-enzyme cascade.

The 3-enzyme cascade worked well at safflower oil concentrations of 30 and 100 mM linoleic acid equivalent (Figure 5c). While the reaction velocity at 30 mM was slightly lower than in the aerated system (Figure 4b), the reaction velocity at 100 mM linoleic acid equivalent was in the same range. A further increase of safflower oil to 300 mM linoleic acid equivalent was possible. The initial reaction velocity was lower, probably caused by non-sufficient emulsification; nevertheless, hydroperoxide yields exceeded 83% after 7 h of reaction. Analysis of the regioisomer pattern revealed that around 90% of 13-hydroperoxides were formed largely independent of the substrate concentration used (Figure 5d). Assuming 83% HPODE yield in total, approximately 70 g/L of 13-HPODE were produced at the highest safflower oil concentration tested with an average synthesis rate of 10 g/L/h. The catalase-based 3-enzyme cascade should be suitable for process intensification to further optimize hydroperoxidation yield and production rates. In accordance combined catalase/oxidase reaction systems were successfully optimized under continuous and pressurized flow conditions [40,41]. In addition, scale-up of the reaction system should be simple and it can be assumed that higher yields may be obtained in larger reactors as was shown before for LOX catalyzed hydroperoxidation [21].





**Figure 5.** (a) Dependence of H<sub>2</sub>O<sub>2</sub> flowrate on LOX-catalyzed hydroperoxidation in the presence of *M. lysodeikticus* catalase at a linoleic acid concentration of 30 mM after 2 h of reaction; (b) Foam evaluation with safflower oil equivalent to 30 mM linoleic acid, top row: 3-enzyme system with catalase and in situ oxygen generation, bottom row: lipase + LOX system with oxygen bubbling; (c) Hydroperoxidation yields with LOX-1 from Sigma, lipase and catalase in Tris HCl buffer pH 8 with ● = safflower oil equivalent to 30 mM, ▲ = 100 mM and ◆ = 300 mM linoleic acid; (d) Analysis of the regioisomer ratio (13:9) of products obtained from reaction shown in (c) with percentage of 13-HPODE (■) and 9-HPODE (▨).

### 3. Materials and Methods

#### 3.1. Materials

LOX-1 and catalase from *Micrococcus lysodeikticus*, and lipases were obtained from Sigma Aldrich (St. Louis, MO, USA). Soybeans were purchased from Wolmersdorf GmbH & Co. KG, Nindorf, Germany, and solvents were obtained from Carl Roth GmbH & Co. KG, Karlsruhe, Germany (ethanol, THF) and Fisher Scientific, Hampton, USA (ethyl acetate). Other chemicals were obtained from VWR International GmbH, Darmstadt, Germany and Sigma Aldrich. Reference standards of 13-Hydroxy-9(Z),11(E)-octadecadienoic acid, 9(R)-hydroxy-10(E),12(Z)-octadecadienoic acid, 13-Hydroperoxy-9(Z),11(E)-octadecadienoic acid and 9-Hydroperoxy-10(E),12(Z)-octadecadienoic acid were purchased from Larodan AB, Solna, Sweden. Mono- and Dilinolein reference standards were obtained from Sigma Aldrich. References of palmitic acid, stearic acid, linoleic acid were purchased from Carl Roth. Oleic acid reference was obtained from Fisher Scientific. Safflower oil was purchased from Gefro KG, Memmingen, Germany and GC analysis showed a content of 77.5% linoleic acid, 13.4% oleic acid, 2.4% stearic acid and 6.7% palmitic acid (Figure S12).

### 3.2. Preparation of Linoleic Acid and Soybean Lipoxygenase

Preparation of linoleic acid from safflower oil was conducted essentially as described before [44]. 1 kg of safflower oil, 250 g of potassium hydroxide, 1 L of ethanol and 1 L of water were mixed in a flask and incubated for 90 min under reflux. After cooling, the mixture was acidified with HCl and the lipid phase was separated, washed and dried. The fatty acid fraction (900 g) was mixed with 900 g of urea and 4.5 L of ethanol in a 10 L vessel and stirred at 70 °C for 1 h to dissolve the urea. Then the solution was crystallized at 8 °C overnight, the precipitate was removed by filtration before the filtrate was diluted with 4.5 L of water and the pH was adjusted to 2.5 with HCl. The upper linoleic acid phase was separated, washed with deionized water and dried under vacuum. According to GC analysis, the enriched linoleic acid contained 93.7% linoleic acid alongside 6.2% oleic acid and 0.1% palmitic acid (Figure S12).

LOX was obtained by freezing soybeans in liquid nitrogen and grounding with a bean mill from Jupiter GmbH, Stuttgart, Germany. The grounded beans were then extracted in 3 volumes of acetone for 90 min. The extraction was conducted three times before residual solvent was removed *in vacuo* at room temperature. Before usage a 10% *w/v* solution of defatted beans in 50 mM borate buffer pH 9 was prepared. The preparation was stirred for 30 min, filtrated and activity was determined photometrically.

### 3.3. Enzyme Activity Measurements and Protein Quantification

Lipoxygenase activity was determined photometrically with an UV-3100 PC Spectrophotometer from VWR International in 50 mM sodium borate buffer pH 9 with 1 mM linoleic acid. Reaction was started by addition of enzyme solution in the cuvette and the increase in absorbance at 234 nm was measured over 5 min. The enzyme activity ( $1 \mu\text{mol}/\text{min} = 1 \text{ U}$ ) was calculated using an extinction coefficient of  $25,000 \text{ M}^{-1} \text{ cm}^{-1}$ .

Catalase activity was analysed photometrically in either 50 mM sodium borate buffer pH 9 or 50 mM Tris-HCl buffer pH 8 with 15 mM hydrogen peroxide. The reaction was started by the addition of enzyme and the consumption of hydrogen peroxide was monitored at 240 nm using an extinction coefficient of  $44.3 \text{ M}^{-1} \text{ cm}^{-1}$ .

Quantification of protein content was done with the Biuret method in triplicate against a bovine serine albumin calibration curve. In a 96-well plate, 100  $\mu\text{L}$  of the appropriate diluted samples were added to 100  $\mu\text{L}$  of a biuret staining solution (0.3 g  $\text{CuSO}_4 \times 5 \text{ H}_2\text{O}$ , 1.21 g NaK-tartrate  $\times 4 \text{ H}_2\text{O}$ , 0.5 g KI diluted in 100 mL 0.2 M NaOH) and incubated in the dark for 30 min. Absorption at 540 nm was measured with a Spectra Max 190 microplate reader from Molecular Devices LLC, San Jose, USA.

### 3.4. Biocatalytic Transformations

Typical LOX experiments were carried out in a volume of 50 mL with continuous oxygen bubbling at a rate of 20–100 mL/min under stirring with either LOX enriched defatted soybean extract or soybean LOX-1 preparation from Sigma. Samples were taken periodically for UV-monitoring of peroxides and after termination of the reaction pH was adjusted to 3.5 and hydroperoxides were extracted with ethyl acetate and washed with water. The remaining solvent was evaporated *in vacuo* using a rotary evaporator at room temperature and the product was used for further analysis. In optimization studies a variety of conditions were tested as outlined in Table 3. Additionally, enzyme and substrate addition over time and the effect of agitation and homogenization methods were examined.

For lipase screening (Table 3), 0.65 mmol safflower oil (MW calculated 874.3 g/mol, 77.5% linoleic acid content, equivalent to a concentration of 30 mM linoleic acid) was homogenized for 30 min with a homogenizer (Kinematica AG Polytron PT 2500 E) in either 50 mL Tris-HCl buffer at pH 8- or 50-mL borate buffer pH 9 at room temperature. The reactions were conducted under stirring and pH control at 20 °C for 5 h with 0.64 mg/mL of technical lipase preparation (5% *w/v* substrate) added. Samples were taken periodically, and hydrolysis was monitored by GC analysis. Combinations of lipase and LOX were done under essentially identical conditions at pH 8. Lipase was always added at the start of the

reaction and LOX was either added at start (100,000 U/mmol substrate) or dosed over the reaction at a flowrate of 10,000 U/mmol substrate per hour.

**Table 3.** Parameters tested for optimization of linoleic acid hydroperoxidation and safflower oil hydrolysis.

Parameter	Range/Compounds
Temperature	20–30 °C
pH-value	6.5–10
Solvents	Methanol, Ethanol, DMSO, MTBE, Acetone, Acetonitrile, Isopropanol, 1-Butanol, Heptane, 1-Octanol
Detergents	Tween 20, Tween 80, Tergitol 15-s-3, Tergitol 15-s-7, Tergitol 15-s-9, Tergitol 15-s-20, Tergitol 15-s-40, Tergitol NP-9, Tergitol NP-10, Ecosurf SA-9, Ecosurf EH-9, Triton X-100, Triton CG-110, Sophorolipid C18:2 [30], Brij 93, Brij O 10, Span 80
Oxygen supply	0 to 100 mL/min
Substrate concentration	1 to 100 mM (linoleic acid equivalent)
Substrate–enzyme ratio	LOX: 1000 to 1,000,000 U/mmol
Lipases	Amano AY 30 ( <i>C. rugosa</i> ) (172.8 ± 33.4 mg/g), Amano A ( <i>A. niger</i> ) (173.9 ± 15.4 mg/g), Amano F-AP 15 (30.8 ± 4.9 mg/g), Amano M ( <i>M. javanicus</i> ) (256.9 ± 11.5 mg/g), Amano ( <i>P. fluorescens</i> ) (109.4 ± 12 mg/g), Amano G ( <i>P. camemberti</i> ) (80.4 ± 5.3 mg/g), Amano PS ( <i>B. cepacia</i> ) (15.1 ± 3.1 mg/g), Lipase CCL ( <i>C. rugosa</i> ) (124.4 ± 17.3 mg/g), Palatase 20,000 L (45.3 ± 4.3 mg/g), Novozymes Lipolase 100 L ( <i>T. lanuginosus</i> ) (13.2 ± 1.4 mg/g), Novozymes Eversa Transform 2.0 (19.8 ± 1.9 mg/g), Novozymes CalB L ( <i>C. antarctica</i> ) (37.8 ± 0.6 mg/g)

The application of catalase for in-situ oxygen generation was analyzed under reaction conditions previously optimized for LOX and the minimum amount of catalase for immediate H<sub>2</sub>O<sub>2</sub> removal over the course of the reaction was determined. LOX and catalase were combined and H<sub>2</sub>O<sub>2</sub> was added with 0–200 mg/(L·min) in 50 mL scale trials. The reaction was followed by hydroperoxide formation and foam build up was monitored visually.

The 3-enzyme-cascade consisting of LOX, lipase and catalase was run under optimized conditions in 50 mL scale at pH 8 in Tris-HCl buffer under stirring and pH control at 20 °C in the presence of Triton CG-110 (3% *w/v* of substrate) for 7 h. 0.65–6.5 mmol safflower oil (equivalent to 30–300 mM linoleic acid), lipase from *Pseudomonas fluorescens* in a concentration of 10% (*w/v* substrate) and 20,000 U catalase from *Micrococcus lysodeikticus* per µmol hydrogen peroxide were added at the start of the reaction. H<sub>2</sub>O<sub>2</sub> dosing was adjusted to 100 mg/(L·min) and LOX-1 dosage was 2000–10,000 U/mmol substrate each hour.

### 3.5. Product Analysis and Quantification

Routine quantification of the hydroperoxidation products was done by analyzing the conjugated double bond system at 234 nm (extinction coefficient of 25,000 M<sup>−1</sup> cm<sup>−1</sup>). Samples were diluted appropriately in ethanol and analyzed with an UV-3100 PC Spectrophotometer from VWR.

Analysis or regioisomers was carried out with a Nexera LC-20AD XR Liquid Chromatograph HPLC from Shimadzu Deutschland GmbH, Duisburg, Germany using a Hitachi (Chiyoda, Japan) LaChrom II C18 RP column (250 mm × 4.6 mm, 5 µm particle size). The injection volume was 10 µL and separation of compounds was achieved using an isocratic flow with 48% acetonitrile:THF:formic acid (67.5:32.5:0.1) and 52% water:formic acid (100:0.1) over 90 min. Peak assignment of 9- and 13-hydroperoxides and hydroxides was done with reference standards. Peak areas of the 9- and 13-hydroperoxides were used for quantification using a calibration curve.

Oil hydrolysis was either analyzed by titration or GC analysis. Lipids were extracted with ethyl acetate after acidifying the sample to pH 3.5 with 1 M HCl. After washing of the solvent phase, ethyl acetate was evaporated under vacuum and the lipid phase was stored at −20 °C until further analysis. 100–300 mg of the lipid phase was diluted with 20 mL anhydrous ethanol and two drops of an ethanolic phenolphthalein solution were added. The lipid phase was titrated against 0.1 M KOH solution under constant stirring

with a Titroline 7000 from SI Analytics Mainz, Germany until the color changed to pink. Acid value was calculated using the following Formula (1).

$$\text{Acid value} = \frac{V_{\text{KOH}}[\text{ml}] \times c_{\text{KOH}}\left[\frac{\text{mol}}{\text{l}}\right] \times M_{\text{KOH}}\left[\frac{\text{g}}{\text{mol}}\right]}{\text{weight}_{\text{sample}}[\text{g}]} \quad (1)$$

Samples for GC analysis were prepared by silylation of the lipid phase with a tenfold excess of BSTFA + TMCS. Silylation was performed by diluting 5 µL of the product sample in 945 µL heptane. 50 µL BSTFA + 1% TMCS solution were added into the vial and silylated at 80 °C for 1 h. Analysis of oil hydrolysis was performed using a Shimadzu GC-2010 system with FID detection and helium as carrier gas with a MTX-Biodiesel TG high-temperature column from Restek GmbH, Bad Homburg, Germany (length 14 m, film thickness 0.16 µm inner diameter 0.53 mm), an injection volume of 1 µL and a split ratio of 1:12. The column temperature at the beginning was set to 75 °C and stepwise increased to 410 °C within 25 min.

Analysis of HPODE via GC was done after hydrogenation of the hydroperoxides. The lipid fraction was extracted with ethyl acetate after acidifying with HCl as described above. 25 µL of lipid sample was dissolved in 500 µL borate buffer pH 9 and 500 µL sodium boron hydride (10 mg/mL) in 1 M NaOH. The mixture was incubated for 1 h at room temperature. After acidification with 5 M HCL, the lipids were extracted in 500 µL MTBE. Silylation was done as described above. GC-Analysis was done using a split ratio of 1:10 and a temperature gradient from 40 °C to 330 °C within 27 min.

Methylation of lipid substrates was performed by weighing in 20 µL of lipid phase and addition of 500 µL boron trichloride in methanol (12% *w/w*). After incubation for 120 min at 60 °C, 500 µL water was added. The lipids were extracted in 1 mL heptane and dried with sodium sulfate. GC-FID analysis was done with an ERAcc-WAX-MS column (length 30 m, film thickness 0.25 µm inner diameter 0.25 mm) from Isera GmbH, Düren, Germany, an injection volume of 1 µL and a split ratio of 1:10. A gradient from 100 °C to 250 °C at a rate of 5 °C/min was applied.

The degree of hydrolysis of hydroperoxide samples obtained from safflower oil was calculated with:

$$\text{Degree of hydrolysis} = \frac{\text{Acid value} \times 100}{\text{Theoretical acid value}} \quad (2)$$

The theoretical acid value was calculated from the average molar mass obtained from compound analysis with GC. The fatty acid composition was analyzed after methylation and the hydroperoxide content after hydrogenation and silylation. A mass of 32 g/mol (O<sub>2</sub>) was added for the hydroperoxide fraction.

#### 4. Conclusions

In this work, we established an enzymatic cascade suitable for one-pot transformation of linoleic acid rich oils into 13-hydroperoxyoctadecadienoic acid in high yield and with good regioselectivity. Foam control with in-situ oxygen production will enable simple scale-up of the reaction system. The system applies well to the “Principles of Green Chemistry” in being solvent-free and using only enzymes at low temperature in the presence of fully biobased surfactant Triton CG-110. Further optimization potentials may target implementation of the 3-enzyme cascade in fixed bed applications or flow systems. Besides immobilization of the enzymes for recycling and cost optimization, cloning of soybean LOX-1 may improve the technical availability of the lipoxygenase.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/catal11091119/s1>, Figure S1: HPLC example chromatogram, Figure S2: Effect of substrate concentration and oxygen flow rate, Figure S3: Influence of homogenization methods, Figure S4: Influence of solvent addition, Figure S5: Influence of HLB on HPODE yield, Figure S6: Lipase screening at pH 8 and 9, Figure S7: Acid value analysis of hydroperoxidation experiments shown in Figure 4a,



Figure S8: Comparison of linoleic acid and monolinolein hydroperoxidation, Figure S9: Comparison of HPODE yield with different LOX dosages. Figure S10: GC chromatograms of hydrogenated samples from experiments shown in Figure 4a, Figure S11: Catalase concentration adjustment; Figure S12: GC-analysis of substrates.

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## References

- Hansen, J.; Garreta, A.; Benincasa, M.; Fusté, M.C.; Busquets, M.; Manresa, A. Bacterial lipoxygenases, a new subfamily of enzymes? A phylogenetic approach. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 4737–4747. [\[CrossRef\]](#) [\[PubMed\]](#)
- Brash, A.R. Lipoxygenases: Occurrence, Functions, Catalysis, and Acquisition of Substrate. *J. Biol. Chem.* **1999**, *274*, 23679–23682. [\[CrossRef\]](#) [\[PubMed\]](#)
- Andreou, A.; Feussner, I. Lipoxygenases Structure and reaction mechanism. *Phytochemistry* **2009**, *70*, 1504–1510. [\[CrossRef\]](#)
- Newcomer, M.E.; Brash, A.R. The structural basis for specificity in lipoxygenase catalysis. *Protein Sci.* **2015**, *24*, 298–309. [\[CrossRef\]](#)
- Andreou, A.; Brodhun, F.; Feussner, I. Biosynthesis of oxylipins in non-mammals. *Prog. Lipid Res.* **2009**, *48*, 148–170. [\[CrossRef\]](#) [\[PubMed\]](#)
- Feussner, I.; Wasternack, C. The lipoxygenase pathway. *Annu. Rev. Plant. Biol.* **2002**, *53*, 275–297. [\[CrossRef\]](#)
- Blée, E. Impact of phyto-oxylipins in plant defense. *Trends Plant. Sci.* **2002**, *7*, 315–322. [\[CrossRef\]](#)
- Vincenti, S.; Mariani, M.; Alberti, J.-C.; Jacopini, S.; De Caraffa, V.B.-B.; Berti, L.; Maury, J. Biocatalytic Synthesis of Natural Green Leaf Volatiles Using the Lipoxygenase Metabolic Pathway. *Catalysts* **2019**, *9*, 873. [\[CrossRef\]](#)
- Nanda, S.; Yadav, J. Lipoxygenase biocatalysis: A survey of asymmetric oxygenation. *J. Mol. Catal. B Enzym.* **2003**, *26*, 3–28. [\[CrossRef\]](#)
- Heshof, R.; De Graaff, L.H.; Villaverde, J.J.; Silvestre, A.; Haarmann, T.; Dalsgaard, T.K.; Buchert, J. Industrial potential of lipoxygenases. *Crit. Rev. Biotechnol.* **2015**, *36*, 665–674. [\[CrossRef\]](#)
- Hayward, S.; Cilliers, T.; Swart, P. Lipoxygenases: From Isolation to Application. *Compr. Rev. Food Sci. Food Saf.* **2016**, *16*, 199–211. [\[CrossRef\]](#) [\[PubMed\]](#)
- Schörken, U.; Kempers, P. Lipid biotechnology: Industrially relevant production processes. *Eur. J. Lipid Sci. Technol.* **2009**, *111*, 627–645. [\[CrossRef\]](#)
- Gigot, C.; Ongena, M.; Fauconnier, M.-L.; Wathélet, J.-P.; du Jardin, P.; Thonart, P. The lipoxygenase metabolic pathway in plants: Potential for industrial production of natural green leaf volatiles. *Biotechnol. Agron. Soc. Environ.* **2010**, *14*, 451–460.
- Gardner, H.W.; Plattner, R.D. Linoleate hydroperoxides are cleaved heterolytically into aldehydes by a Lewis acid in aprotic solvent. *Lipids* **1984**, *19*, 294–299. [\[CrossRef\]](#) [\[PubMed\]](#)
- Kerler, J.; Kohlen, E.; Fitz, W.; Vliet, A.V.D.; Winkel, C. Method for the Enzymatic Preparation of Aldehydes Rich Aromas c6-c10. U.S. Patent No. 6864072, 8 March 2005.
- Otte, K.B.; Kirtz, M.; Nestl, B.M.; Hauer, B. Synthesis of 9-Oxononanoic Acid, a Precursor for Biopolymers. *ChemSusChem* **2013**, *6*, 2149–2156. [\[CrossRef\]](#)
- Löwe, J.; Dietz, K.; Gröger, H. From a Biosynthetic Pathway toward a Biocatalytic Process and Chemocatalytic Modifications: Three-Step Enzymatic Cascade to the Plant Metabolite cis-(+)-12-OPDA and Metathesis-Derived Products. *Adv. Sci.* **2020**, *7*, 1902973. [\[CrossRef\]](#) [\[PubMed\]](#)
- Baysal, T.; Demirdöven, A. Lipoxygenase in fruits and vegetables: A review. *Enzym. Microb. Technol.* **2007**, *40*, 491–496. [\[CrossRef\]](#)
- Drouet, P.; Thomas, D.; Legoy, M.D. Production of 13(S)-hydroperoxy-9(Z),11(E)-octadecadienoic acid using soybean lipoxygenase 1 in a biphasic octane-water system. *Tetrahedron Lett.* **1994**, *35*, 3923–3926. [\[CrossRef\]](#)
- Márczy, J.S.; Németh, Á.S.; Samu, Z.; Hager-Veress, Á.; Szajáni, B. Production of hexanal from hydrolyzed sunflower oil by lipoxygenase and hydroperoxid lyase enzymes. *Biotechnol. Lett.* **2002**, *24*, 1673–1675. [\[CrossRef\]](#)
- Fauconnier, M.L.; Marlier, M. An efficient procedure for the production of fatty acid hydroperoxides from hydrolyzed flax seed oil and soybean lipoxygenase. *Biotechnol. Tech.* **1996**, *10*, 839–844. [\[CrossRef\]](#)
- Németh, Á.S.; Márczy, J.S.; Samu, Z.; Hager-Veress, Á.; Szajáni, B. Biocatalytic production of 2(E)-hexenal from hydrolyzed linseed oil. *Enzym. Microb. Technol.* **2004**, *34*, 667–672. [\[CrossRef\]](#)
- Piazza, G. Lipoxygenase catalyzed hydroperoxide formation in microemulsions containing nonionic surfactant. *Biotechnol. Lett.* **1992**, *14*, 1153–1158. [\[CrossRef\]](#)
- Rodakiewicz-Nowak, J.; Maślakiewicz, P.; Haber, J. The effect of Linoleic Acid on pH Inside Sodium bis(2-ethylhexyl) sulfosuccinate Reverse Micelles in Isooctane and on the Enzymic Activity of Soybean Lipoxygenase. *J. Biol. Inorg. Chem.* **1996**, *238*, 549–553. [\[CrossRef\]](#)
- Kermasha, S.; Dioum, N.; Bisakowski, B. Biocatalysis of lipoxygenase in selected organic solvent media. *J. Mol. Catal. B Enzym.* **2001**, *11*, 909–919. [\[CrossRef\]](#)

26. Gargouri, M.; Legoy, M.D. Biezymatic reaction for hydroperoxide production in a multiphasic system. *Enzym. Microb. Technol.* **1997**, *21*, 79–84. [\[CrossRef\]](#)
27. Gargouri, M.; Legoy, M.D. A two-enzyme system for the transformation of unsaturated oils to 9(S)-hydroperoxy fatty acids. *Biotechnol. Lett.* **2002**, *24*, 915–918. [\[CrossRef\]](#)
28. Wang, J.; Li, K.; He, Y.; Liu, X.; Wang, P.; Xu, L.; Yan, J.; Yan, Y. Bi-enzyme directed self-assembled system toward biomimetic synthesis of fatty acid hydroperoxides like soybean. *Compos. Part. B: Eng.* **2021**, *222*, 109091. [\[CrossRef\]](#)
29. Kim, K.-R.; An, J.-U.; Lee, S.-H.; Oh, D.-K. Selective Production of 9R-Hydroxy-10E,12Z,15Z-Octadecatrienoic Acid from  $\alpha$ -Linolenic Acid in Perilla Seed Oil Hydrolyzate by a Lipoyxygenase from Nostoc Sp. SAG 25.82. *PLoS ONE* **2015**, *10*, e0137785. [\[CrossRef\]](#)
30. Elshof, M.; Janssen, M.; Veldink, G.; Vliegthart, J. Biocatalytic large-scale production of 13(S)-hydroperoxy-9(Z), 11(E) octadecadienoic acid from hydrolysed safflower oil by a crude soybean-flour extract as lipoyxygenase source. *Recl. Trav. Chim. Pays-Bas* **1996**, *115*, 499–504. [\[CrossRef\]](#)
31. Berry, H.; Debat, H.; Larreta-Garde, V. Excess substrate inhibition of soybean lipoyxygenase-1 is mainly oxygen-dependent. *FEBS Lett.* **1997**, *408*, 324–326. [\[CrossRef\]](#)
32. Griffin, W.C. Calculation of HLB values of non-ionic surfactants. *Soc. Cosmet. Chem.* **1954**, *5*, 249–256.
33. Von Rybinski, W.; Hill, K. Alkyl polyglycosides—Properties and applications of a new class of surfactants. *Angew. Chem. Int. Ed.* **1998**, *37*, 1328–1345. [\[CrossRef\]](#)
34. Heo, S.-H.; Hou, C.T.; Kim, B.S. Production of oxygenated fatty acids from vegetable oils by Flavobacterium sp. strain DS5. *New Biotechnol.* **2009**, *26*, 105–108. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Jiang, Y.; Li, Z.; Zheng, S.; Xu, H.; Zhou, Y.J.; Gao, Z.; Meng, C.; Li, S. Establishing an enzyme cascade for one-pot production of  $\alpha$ -olefins from low-cost triglycerides and oils without exogenous H<sub>2</sub>O<sub>2</sub> addition. *Biotechnol. Biofuels* **2020**, *13*, 1–11. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Jeon, E.-Y.; Seo, J.-H.; Kang, W.-R.; Kim, M.-J.; Lee, J.-H.; Oh, D.-K.; Park, J.-B. Simultaneous Enzyme/Whole-Cell Biotransformation of Plant Oils into C9 Carboxylic Acids. *ACS Catal.* **2016**, *6*, 7547–7553. [\[CrossRef\]](#)
37. Gargouri, M.; Legoy, M.D. Chemoenzymatic production of (+)-coriolic acid from trilinolein: Coupled synthesis and extraction. *J. Am. Oil Chem. Soc.* **1997**, *74*, 641–645. [\[CrossRef\]](#)
38. Stolterfoht, H.; Rinnofner, C.; Winkler, M.; Pichler, H. Recombinant Lipoyxygenases and Hydroperoxide Lyases for the Synthesis of Green Leaf Volatiles. *J. Agric. Food Chem.* **2019**, *67*, 13367–13392. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Schneider, K.; Dorscheid, S.; Witte, K.; Giffhorn, F.; Heinzle, E. Controlled feeding of hydrogen peroxide as oxygen source improves production of 5-ketofructose from L-sorbose using engineered pyranose 2-oxidase from *Peniophora gigantea*. *Biotechnol. Bioeng.* **2012**, *109*, 2941–2945. [\[CrossRef\]](#)
40. Bolivar, J.M.; Mannsberger, A.; Thomsen, M.S.; Tekautz, G.; Nidetzky, B. Process intensification for O<sub>2</sub>-dependent enzymatic transformations in continuous single-phase pressurized flow. *Biotechnol. Bioeng.* **2018**, *116*, 503–514. [\[CrossRef\]](#)
41. Chapman, M.R.; Cosgrove, S.C.; Turner, N.J.; Kapur, N.; Blacker, A.J. Highly Productive Oxidative Biocatalysis in Continuous Flow by Enhancing the Aqueous Equilibrium Solubility of Oxygen. *Angew. Chem. Int. Ed.* **2018**, *57*, 10535–10539. [\[CrossRef\]](#)
42. Beverungen, C.; Buyukuslu, N.; Candar, V.; Eryasa, Y.; Schörken, U.; Weiss, A.; Wunderlich, M. Process for recovering hydroperoxides. European Patent application EP 1336659A2, 7 February 2003.
43. Pérez-Gilabert, M.; Veldink, G.A.; Vliegthart, J.F.G. Protection by different agents against inactivation of lipoyxygenase by hydrogen peroxide. *Lipids* **1996**, *31*, 1245–1250. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Bollmann, T.; Zerhusen, C.; Glösen, B.; Schörken, U. Structures and Properties of Sophorolipids in Dependence of Microbial Strain, Lipid Substrate and Post-Modification. *Tenside Surfactants Deterg.* **2019**, *56*, 367–377. [\[CrossRef\]](#)