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Dual Positional and Stereospecificity of Lipoxygenase Isoenzymes from Germinating Barley (Green Malt): Biotransformation of Free and Esterified Linoleic Acid

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The lipoxygenase isoenzymes LOX1 and LOX2 from green malt were separated by isoelectric focusing, and their catalytic properties regarding complex lipids as substrates were characterized. The regio- and stereoisomers of hydroperoxy octadecadienoates (HPODE) resulting from LOX1 and LOX2 enzymatic transformations of linoleic acid, methyl linoleate, linoleic acid glycerol esters monolinolein, dilinolein, and trilinolein, and 1-palmitoyl-2-linoleoyl-glycero-3-phosphocholine (PamLinGroPCho) were determined. In addition, biotransformations of polar and nonpolar lipids extracted from malt were performed with LOX1 and LOX2. The results show that LOX2 catalyzes the oxidation of esterified fatty acids at a higher rate and is more regioselective than LOX1. The dual position specificity of LOX2 (9-HPODE:13-HPODE) with trilinolein as the substrate (6:94) was higher than the resultant ratio (13:87) when free linoleic acid was transformed. A high (*S*)-enantiomeric excess of 13-HPODE was analyzed with all esterified substrates confirming the formation of 13-HPODE through the LOX2 enzyme; however, 9-HPODE detected after LOX2 biotransformations showed (*R*)-enantiomeric excesses. PamLinGroPCho was oxygenated by LOX1 with the highest regio- and stereoselectivities among the applied substrates.

KEYWORDS: Lipoxygenase; barley; green malt; HPODE; regioselectivity; stereoselectivity

INTRODUCTION

Lipoxygenases (oxidoreductase, EC 1.13.11.12, LOX) catalyze the peroxidation of polyunsaturated fatty acids containing a 1*Z*,4*Z*-pentadiene system to yield the (*S*)-configured hydroperoxy fatty acids. These in turn can be converted through nonenzymatic or enzymatic reactions leading to a variety of secondary metabolites including compounds that are considered to play a role in staling and off-flavor formation in beer (*1*, *2*).

First described in plants as lipoxidase (*3*), LOX has been the subject of extensive studies (*4*−*6*). The LOX pathway in mammalian systems has been related to the formation of regulatory compounds that play a role in inflammation, immunity, hypersensitivity, and host defense reactions (*7*). The physiological function of LOX in plants is not completely understood, but participation in plant senescence (*8*), environmental stress responses (*9*), and lipid mobilization during seed germination (*10*) has been suggested.

Many isoenzymes of LOX have been found in plants. In germinating barley, two LOX isoenzymes called LOX1 and LOX2 are described (*11*, *12*). While LOX1 is already present in barley and catalyzes the formation of (9*S*)-9-hydroperoxy-10*E*,12*Z*-octadecadienoic acid [(*S*)-9-HPODE], LOX2 is formed during germination and catalyzes the formation of (13*S*)-13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid [(*S*)-13-HPODE] with linoleic acid as the substrate (*12*−*14*).

Although representing the best substrates for both LOX isoenzymes (*15*), free linoleic and linolenic acid constitute only about 6% of the total fatty acid content in barley (*16*). About 70% of the fatty acids of barley and malt have been found as triglycerides and approximately 20% as polar lipids (phospholipids and glycolipids) (*17*).

Besides favoring the yeast performance during fermentation, lipids can negatively influence filtration rates, decrease foam stability, and may give rise to the development of off-flavor compounds (*14*). In the literature, much information can be found about the behavior of LOX isoenzymes in the presence of free fatty acids (*18*), but only little work was done with esterified fatty acids. The lipid content in malt (3.4% dry matter) is lower than in barley (4.4%) showing that some lipid is degraded during the malting process (*19*). Relative activities of LOX1 and LOX2 as compared to linoleic acid using methyl linoleate (Me-Lin), 1,3-dilinoleoyl-glycerol (dilinolein), 1,2,3-trilinoleoyl glycerol (trilinolein), and 1-palmitoyl-2-linoleoyl-glycero-3-phosphocholine (PamLinGroPCho) as substrates were determined by Holtman et al. (*13*). Both isoenzymes were able

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to metabolize more complex substrates; however, LOX2 exhibited higher relative activities than LOX1. The kinetic parameters for both isoenzymes (V_{max}; K_m) were investigated (*13*), and LOX2 showed a higher metabolic capacity for esterified fatty acids than LOX1.

In barley and malt, the major part of the hydroxy fatty acids [hydroxy octadecadienoic acid (HODE)] is esterified as triacylglycerols (*22*); however, 35% of total HODE are found in polar lipids (*21*).

Characterization of triacylglycerol-esterified HODE in barley and in the correspondent 6 day green malt showed an increase of (*S*)-9-HPODE and (*S*)-13-HPODE with a ratio of 17:83 after 6 days of germination. This ratio could be due to LOX2 regioselectivity. The increase of the amounts of (*R*)-9-HPODE and (*R*)-13-HPODE after 6 days of germination in a ratio of 14:86 indicated an autoxidation with simultaneous degradation of (*R*)-9-HODE via β-oxidation (*20*). Regio- and stereoisomers of 9- and 13-HODE in barley, green malt, and final malt have been used as parameters to study the catalysis of LOX in the presence of free and esterified fatty acids. In addition, further factors such as autoxidation, the action of the hydroperoxyde-metabolizing enzymes (HPME), and lipases can affect the amount of HPODE as well as their regio- and stereoisomer ratios catalyzed by LOX in the presence of complex lipid substrates.

The present paper describes the biotransformation of free linoleic acid and complex esterified lipids performed with green malt LOX isoenzymes and the characterization of all resulting HPODE regio- and stereoisomers.

Free linoleic acid, commercial available esters such as Me-Lin, monolinolein, dilinolein, trilinolein, and PamLinGroPCho, and the extracted polar and nonpolar lipid fractions from malt were used as substrates. Their biotransformation was performed utilizing partially purified and completely separated green malt LOX isoenzymes obtained by isoelectric focusing (IEF). Regio- and stereoisomers of HPODE were characterized by gas chromatography−electron impact mass spectrometry (GC-EI/MS) and chiral high-performance liquid chromatography (HPLC) analysis.

MATERIALS AND METHODS

Preparation of Green Malt for LOX Extraction. Eight boxes containing 400 g of barley samples each were steeped to 44% moisture content at 16 °C. Two air rests were included for each 22 h of steep until reaching 44% moisture. Germinations were conducted at 16 °C maintaining the same barley moisture with the addition of distilled water. After 5 days of germination, samples were removed and stored at −16 °C until enzyme extraction.

LOX Extraction and Dialysis. LOX extracts were prepared according to the method of Lulai and Baker (*23*). All steps were performed at 4 °C. The ground green malt was mixed with 0.1 M phosphate buffer containing 0.7 mM phenylmethylsulfonylfluorid (PMSF), pH 6.0, for 1 h, and swirling for 30 s every 15 min. The extraction ratio [buffer:green malt (dry matter)] was 24:1. Afterward, the liquid was centrifuged at 15000*g* for 15 min and the supernatant (crude enzyme extract) was separated. A 140 mL amount of the crude enzyme extract was saturated (20%) with ammonium sulfate and centrifuged at 12000*g* for 10 min. The obtained pellet was discarded, and the supernatant was precipitated with ammonium sulfate at 50% saturation by centrifugation at 12000*g* for 10 min. The pellet was resuspended in 4 mL of 10 mM potassium phosphate buffer, pH 6.0, and dialyzed (12 kDa MWCO) for 12 h against 0.01 M phosphate buffer, pH 6.0.

IEF of LOX1 and LOX2 and HPME. LOX1 and LOX2 were purified/separated using preparative IEF. The dialyzed LOX extracts were submitted to IEF using the Rotofor apparatus (BIO-RAD) in a 8% solution ampholyte, pH 5−8, according to manufacturer's instructions. Focusing was performed for 6 h at 12 W of constant power at 4 °C. The focused samples obtained at pH 5.4 (LOX1) and pH 6.7 (LOX2) corresponded to the partially purified isoenzymes. In total, eight extractions of each 8.36 g of green malt (dry matter) were subjected to focusing. Eight enzyme extract preparations were necessary to obtain enough enzyme amount for one IEF. Two IEF runs were performed to achieve approximately 1000 mU of each LOX isoenzyme. IEF fractions were also incubated with (*S*)-9- and (*S*)-13-HPODE to characterize (*S*)-HPODE-metabolizing enzyme activities (HPME) by measuring the decrease of 234 nm absorption. No significant HPME activities were determined within the LOX active fractions (*18*).

LOX Enzyme Assay. The LOX activity was measured spectrophotometrically at 234 nm as described previously (*15*). The linoleic acid solution (substrate) was prepared according to the method of Surrey (*24*). One unit of LOX was expressed as the rate of formation of conjugated diene chromophore (1 μmol of hydroperoxide formed per minute) at 25 °C using a molar absorption coeffient of 25000 M^{-1} cm^{-1}.

Substrate Preparation of Esterified Linoleic Acid. Me-Lin, monolinolein [1-monolinoleoyl-glycerol (Lin1Gro)], dilinolein [1,3-dilinoleoyl-glycerol (Lin2Gro)], trilinolein [(1,2,3-trilinoleoyl glycerol) (Lin3Gro)], and PamLinGroPCho were used as substrates. Stock emulsions were prepared by emulsification of the esterified lipids in 0.1 M potassium phosphate puffer, pH 6.5 (degassed), containing 1% Tween 20 with a sonifier (ultrasound) followed by stirring under argon atmosphere. The final linoleic acid equivalent concentration of each substrate in the stock emulsions was 8 mM. The stock emulsions were prepared on the same day of use.

Substrate Preparation of Extracted Polar and Nonpolar Lipids. After the grains were milled [180 g (6 × 30 g)], the nonpolar fraction of malt was extracted in a Soxhlet apparatus (16 h) using *n*-pentane. The solvent was evaporated, and the lipids were applied to a silica gel 60 column chromatography using petroleum ether:ethyl acetate (PE:EA) as the eluent [90 + 10 (v + v)]. The purification step enabled the separation of nonpolar lipids from preoxidized lipids and free fatty acids due to their different polarity. The LC was monitored by thin-layer chromatography using the same eluent ratio and sodium molybdate as the revelatory solution. Nonpolar lipid-containing fractions were pooled and utilized for the preparation of stock emulsions; the amount and fatty acid composition were estimated as described by Schulte et al. (*25*). The polar lipid fraction of malt was obtained from the filter cake after Soxhlet extraction (16 h). Under nitrogen, the filter cake was suspended in 600 mL of $CHCl_3$:MeOH (2:1) and 100 mg of 2,6-bis-(*tert*-butyl)-4-methylphenol and heated to reflux for 15 min. The slurry was filtrated under nitrogen, the solvent was evaporated, and the residue was dissolved in water (30 mL) and extracted with pentane (2 × 20 mL). The pentane phase was discharged. The aqueous phase was lyophilized, and the remaining extracted polar lipids were used to prepare the stock emulsions. The lipid amount and fatty acid composition were analyzed as described previously (*25*).

Stock emulsions containing the extracted nonpolar lipids or the extracted polar lipids were prepared on the same day of use as described for the commercial available linoleic acid esters.

Biotransformations. Transformations with purified LOX from IEF were performed for 2 h at room temperature in a 100 mL Erlenmeyer flask with a constant O_2 supply. The reaction mixture contained 4 mL of the substrate solution (8 mM linoleic acid) and 1000 mU of purified LOX1 or LOX2 isoenzyme. The final volume of the reaction (15 mL) was reached by addition of 0.1 M O_2-saturated phosphate buffer, pH 6.5. The final linoleic acid equivalent concentration in the mixture was 2.12 mM for all substrates. Blank samples (autoxidation) were prepared at the same conditions with the difference of utilizing 0.1 M phosphate buffer, pH 6.5, instead of the enzyme solution with each substrate. Each enzyme preparation and biotransformation was performed in duplicate, and results are given as the average of two independent experiments.

Lipases. To exclude lipase activities in IEF-separated and -purified LOX fractions from green malt, PamLinGroPCho, Lin3Gro, Lin2Gro, and extracted nonpolar lipids from malt (ENPL) were tested as substrates for possible lipase actions in the IEF-purified LOX1 and LOX2 fractions. According to the LOX biotransformations 2.12 mM PamLinGroPCho, Lin3Gro, Lin2Gro, and ENPL were incubated with

IEF fractions under nitrogen atmosphere for 2 h. The aqueous phases were acidified by addition of 1 M HCl (pH 2), extracted twice each with 50 mL of diethyl ether, methylated with diazomethane (*20*), evaporated, and analyzed by GC-MS. Commercial authentic fatty acid methyl ester standards were used as references.

Quantification of HPODE. The amount of isomeric 9-HPODE and 13-HPODE resulting from the reaction of LOX1 or LOX2 with the correspondent substrates as well as the amount resulting from the reactions performed without enzyme (blank sample) were quantified as follows: After biotransformation, 12-monohydroxystearic acid was added (internal standard, extraction, and response factors were determined previously). The sample was extracted with $CHCl_3$:MeOH 2:1 and reduced with $NaBH_4$ (*27*). The solution containing the reduced esterified hydroxy fatty acids was acidified to pH 3 with 1 N HCl and extracted with $CHCl_3$:MeOH 2:1 (extracted polar lipids and PamLin-GroPCho) or with diethyl ether (free and esterified linoleic acid and extracted nonpolar lipids). The organic layer was washed with brine until neutral reaction and dried over Na_2SO_4. The solvent was evaporated, and the residue was saponified (20 mL of MeOH + 15 mL of 40% NaOH/MeOH) at 60 °C during 40 min under nitrogen. After saponification, the solution was acidified to pH 3 with 2 M phosphoric acid and extracted with ethyl acetate (3 × 50 mL). The organic layer was washed with brine until neutral reaction and dried over Na_2SO_4. The solvent was evaporated, and the residue was methylated with diazomethane (*26*), hydrogenated (PtO_2/H_2) (*28*), and silylated (*26*). The obtained trimethylsilylated methyl hydroxystearate derivatives were analyzed by GC-MS. GC was performed on a HP 5890 Series II Gas chromatograph with a HP1 column [Agilent Technologies, Palo Alto, CA (formerly Hewlett-Packard)]. The injection volume was 0.5 μL. The temperature program was as follows: initial temperature, 80 °C; final temperature, 280 °C; and temperature rate, 4 °C/min. Helium N50 was used as the carrier gas. Mass spectra were recorded with a HP 5970 Series Mass Selective Detector. The data were processed with HP MSD ChemStation software.

Relative Activity of LOX. Relative activities of LOX isoenzymes obtained by IEF were determined as follows: The total amounts of 9-HPODE and 13-HPODE resulted from the 2 h of enzymatic reactions that were was used as parameters to determine the enzymatic catalysis rate of the isoenzymes for each substrate. The obtained amount of HPODE after biotransformation of the esterified substrates was compared with the detected amount when linoleic acid was used as the substrate (set to 100%). The total amount of HPODE in the blank sample (transformation without enzyme) was used to estimate the autoxidation of the substrate occurred during biotransformation; these amounts were subtracted. A spectrophotometrical UV test was not sensitive for all substrates.

Chiral Analysis of HODE. (*R*)- and (*S*)-9- and 13-HODE ratios were determined by chiral phase HPLC of their corresponding methyl esters. Standards of (*S*)- and (*R*,*S*)-9- and 13-HODE were obtained as described (*20*). 9-HODE-Me and 13-HODE-Me from biotransformations or blank samples were separated on a normal phase semipreparative HPLC column [Merck Hibar RT 250−1000 Si60 (5 μm), Merck, Darmstadt, Germany] using hexane/diethyl ether (70:30) as the mobile phase with a flow rate of 3 mL/min. (*R*)- and (*S*)-enantiomers of 9-HODE and 13-HODE were separated on a chiral HPLC column (Chiralcel OD-H Daicel Chemical Industries, CHIRACEL Europe, Illkirch, Cedex, France), using hexane/2-propanol 98:2 (v:v) as the mobile phase at a flow rate of 1 mL/min according to the method of Martini et al. (*29*). Products were detected with a UV detector (Merck Hitachi L-7400) at 235 nm; *S*:*R* were calculated from peak areas.

RESULTS

Purification of LOX Isoenzymes from Green Malt. Preparative IEF enabled the separation and isolation of LOX isoenzymes from ground malt. The first peak containing LOX1 showed an isoelectric point at pH 5.4 while the second peak (LOX2) showed an isoelectric point at pH 6.7; however, the LOX enzymes were not purified to homogeneity. To exclude enzymatic activities, which would affect the isomer ratio,

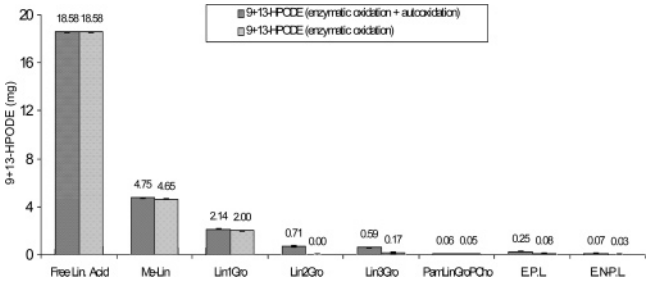

Figure 1. Total HPODE (mg) amount after 2 h of reaction with isoelectric-focused LOX1 enzyme (1000 mU) with different lipids containing the same molarity of linoleic acid at pH 6.5. The confidence interval was calculated at a 95% level with 7 degrees of freedom ($n = 2$, $t = 2.365$).

hydroperoxide-metabolizing activities and lipase activities were tested in the IEF LOX fractions. Therefore, 9-hydroperoxide-metabolizing enzyme (9-HPME) activities and 13-hydroperoxide-metabolizing enzyme (13-HPME) activities were determined by incubating linoleic acid, (9*S*)-9-HPODE, or (13*S*)-13-HPODE with IEF-separated isoenzyme fractions and measuring the increase (linoleic acid) or decrease (HPODE) of 234 nm UV absorption. HPODE-degrading enzymes, which are determined by this method, are, e.g., HPODE isomerase, HPODE lyase, and allene oxide synthase (AOS). Peroxidase activity, which is not detected by this method, would not interfere with the regioisomer ratio. The HPME method showed a very weak hydroperoxide-metabolizing activity, which did not affect the regioisomer ratio (*18*). The action of lipases was investigated by incubation of IEF-separated LOX1 and LOX2 fractions with the representative phospholipid and glycerol esters PamLin-GroPCho, Lin3Gro, Lin2Gro, and ENPL under nitrogen. The lipase test resulted in no liberation of free fatty acids from the applied representative substrates; therefore, lipase enzymatic action in the IEF-separated LOX fractions can be excluded.

Biotransformations of Free and Esterified Linoleic Acids by LOX1 and LOX2. Substrate specificity studies indicated both LOX1 and LOX2 as catalysts to oxygenate free fatty acids as well as esterified lipids. Resulting HPODEs were analyzed as trimethylsilyl (TMS) ethers of methyl hydoxyoctadecanoates by GC-MS and as methyl hydroxyoctadecadienoates by HPLC. The amounts of 9- and 13-HPODE after LOX1 and LOX2 biotransformations of free and esterified linoleic acids are shown in **Figures 1** and **2**, respectively. Although the total active amounts of LOX1 and LOX2 isoenzymes were the same for all experiments (1000 mU), the enzymatic transformations performed with LOX2 (Figure 2) yielded higher amounts of HPODE for all esterified substrates. Apparently, LOX2 has a higher affinity for esterified fatty acids than LOX1. Significant amounts of all substrates still remained after 2 h of biotransformation (data not shown) indicating that substrate limitation did not affect the enzymatic product formation.

LOX1. Among the esterified linoleic acid substrates, methyllinoleate, followed by monolinolein, exhibited the highest transformation rate. A 4.65 mg amount of HPODE was formed during the reaction of methyllinoleate with LOX1 while 2 mg of HPODE was formed from monolinolein. No enzymatic formation of HPODE from dilinolein was analyzed, only autoxidation. The blank sample of trilinolein formed 0.42 mg of HPODE by autoxidation; however, the enzymatic transformation of this substrate by LOX1 yielded an additional 0.17 mg of HPODE. In contrast to the literature where no autoxidation products were formed (*13*), significant amounts of HPODE were detected when dilinolein and trilinolein were incubated with borate buffer at pH 6.5 and oxygen. Low amounts of HPODE

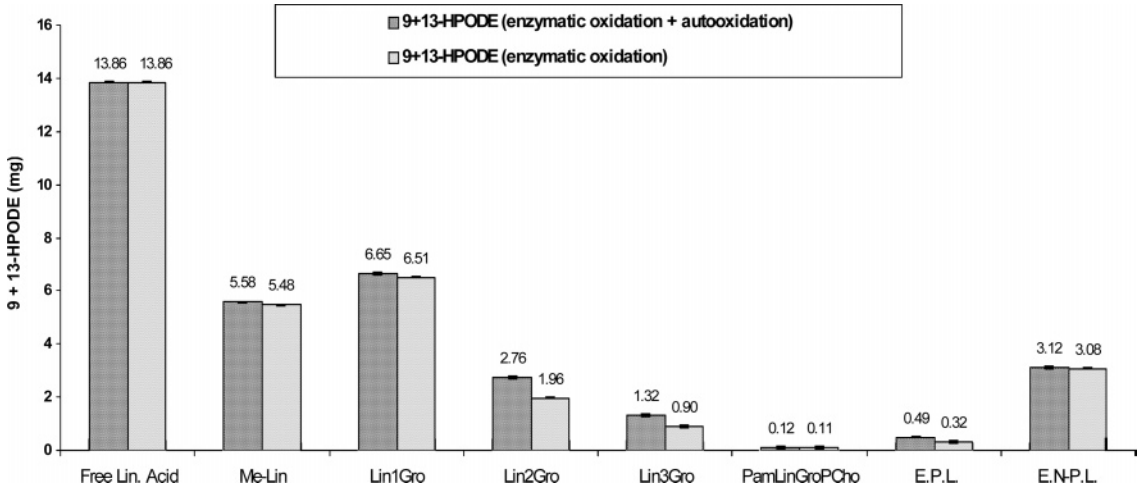

Figure 2. Total HPODE (mg) amount after 2 h of reaction with isoelectric-focused LOX2 enzyme (1000 mU) with different lipids containing the same molarity of linoleic acid at pH 6.5. The confidence interval was calculated at a 95% level with 7 degrees of freedom ($n = 2$, $t = 2.365$).

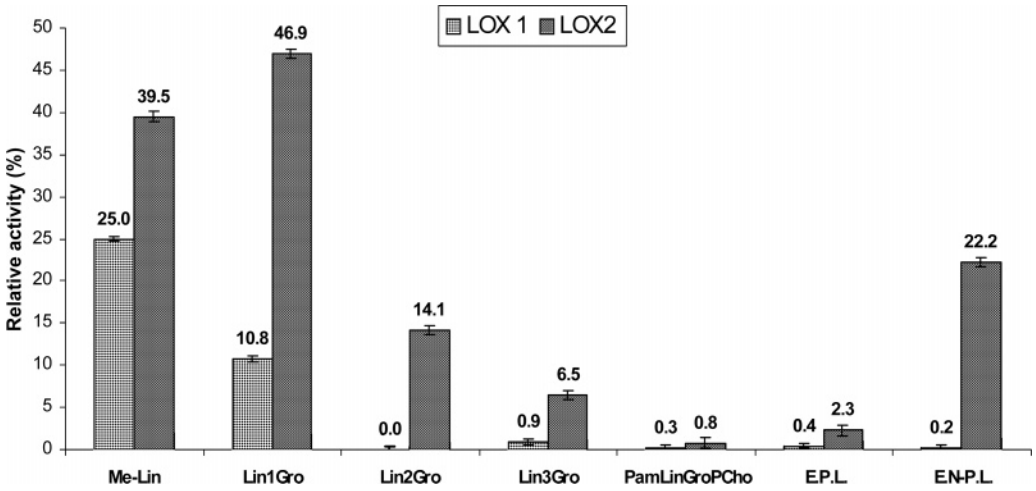

Figure 3. LOX1 and LOX2 activities using esterified linoleic acids as substrates as compared to linoleic acid (set at 100%). Activities were estimated based on the amount of formed HPODE (9- and 13-HPODE) after 2 h of reaction using 2.12 mM linoleic acid equivalent of each substrate. The confidence interval was calculated at a 95% level with 7 degrees of freedom ($n = 2$, $t = 2.365$).

were obtained after 2 h of LOX1 biotransformation when PamLinGroPCho (0.05 mg), extracted polar lipids (0.08 mg), and ENPL (0.03 mg) were used.

LOX2. As shown in Figure 2, 6.51 mg of HPODE was detected after 2 h of LOX2 biotransformation of monolinolein. Using methyllinoleate as the substrate, 5.48 mg of HPODE was formed. Apparently, the autoxidation of monolinolein and Me-Lin did not interfere with the quantification of enzymatically formed HPODE. The HPODE formation by autoxidation of dilinolein and trilinolein was significant; however, the enzymatic catalysis with these substrates is sufficient for analysis of HPODE (1.96 and 0.90 mg, respectively). The amount of HPODE formed by LOX2 from trilinolein (Figure 2) was approximately five times higher than formed by LOX1 (**Figure 1**). LOX2 reaction with extracted polar lipids led to a higher HPODE amount (3×) than for PamLinGroPCho. The use of extracted nonpolar lipids as substrate for LOX2 resulted in a high amount of HPODE (3.08 mg), which was approximately 100-fold higher than that catalyzed by LOX1.

Relative Activities of LOX Isoenzymes. The relative activities of LOX1 and LOX2 toward esterified linoleic acids as compared to linoleic acid are shown in **Figure 3**. The relative activities were determined by analysis of HPODE amount formation. Significant amounts of the substrates were still remaining after the biotransformations. Therefore, substrate limitation did not affect the product formation by LOX1 and LOX2 and among the different substrates. Linoleic acid proved to be the preferred substrate for both isoenzymes. The relative LOX1 activity with Me-Lin and monolinolein as substrates was 25 and 10.8%. LOX1 showed only residual relative activities (<1%) with dilinolein, trilinolein, PamLinGroPCho, extracted polar lipids from malt (EPL), and ENPL. The relative LOX2 activities with Me-Lin and monolinolein were 39.5 and 46.9%, respectively. More complex substrates such as dilinolein, trilinolein, and PamLinGroPCho were transformed by LOX2 with relative activities of 14.1, 6.5, and 0.8%. These results show that LOX2 catalyzed the oxidation of esterified derivatives of linoleic acid at a much faster rate than LOX1. EPLs were also faster oxidized by LOX2 (2.3% relative activity) than by LOX1 (0.4%). LOX2 showed 22.2% relative activity for ENPL while for LOX1 0.2% were detected (**Figure 3** and **Table 2**). Holtman et al. (*13*) and Yang et al. (*15*) reported higher relative rates for

Table 1. Fatty Acid Composition of Extracted Lipids from Malt

	acid (%)				
	palmitic	stearic	oleic	linoleic	linolenic
extracted polar lipids	36.98	5.13	7.35	44.98	5.56
extracted nonpolar lipids	18.2	1.34	12.07	63.25	5.14

Table 2. Relative Activities of LOX Isoenzymes with Lipid Substrates, Resulting 9-:13- Regioisomer Ratios and Enantiomeric Excesses of HPODE Stereoisomers

	LOX1 9-HPODE : 13-HPODE			LOX2 9-HPODE : 13-HPODE	
Free Lin. Acid	9 86(S)	1 4(R)	Free Lin. Acid	1 20(R)	7 78(S)
Me-Lin	3.9 78(S)	1 4(S)	Lin1Gro	1 4(S)	2.6 70(S)
Lin1Gro	2.1 74(S)	1 52(S)	Me-Lin	1 16(R)	4.9 78(S)
Lin3Gro	1.6 12(R)	1 14(S)	E.N-P.L.	1 20(R)	13.7 82(S)
E.P.L.	1 10(S)	1.7 14(S)	Lin2Gro	1 16(R)	8.7 68(S)
PamLinGroPCho	20 84(S)	1 50(S)	Lin3Gro	1 18(R)	14.6 64(S)
E.N-P.L.	1 12(S)	1.1 10(S)	E.P.L.	1 6(R)	51.6 84(S)
Lin2Gro	--- 6(S)	--- 4(S)	PamLinGroPCho	1 ---	8.3 86(S)

Relative activity

biotransformations of methyllinoleate, dilinolein, trilinolein, and PamLinGroPCho.

Regioselectivity of LOX Isoenzymes with Commercial Substrates. The HPODE regioisomer ratios were determined as TMS ethers of methyl hydroxyoctadecanoates by GC-EI/MS. The calculated amounts of the HODE regioisomers represent the regioselectivity of the LOX isoenzymes. Chemical autoxidation occurring during the incubation was estimated by use of blank samples, and these amounts of HODE were subtracted.

LOX1. The results of the regioselectivity of LOX1 with free and esterified linoleic acids are shown in **Figure 4**. LOX1 catalyzed the formation of 9-HPODE:13-HPODE in a ratio of 79:21, 68:32, and 62:38 with methyllinoleate, monolinolein, and trilinolein as substrates. The regioselectivity of LOX1 was higher when linoleic acid was used (90:10); however, a regioselectivity of 95:5 resulted when PamLinGroPCho was used as the substrate (**Figure 4**).

LOX2. The LOX isoenzyme LOX2 exhibited higher regioselectivities than LOX1 in the presence of all esterified lipid substrates except for PamLinGroPCho (11:89). LOX2-catalyzed regioisomer ratios of HPODE with Me-Lin, monolinolein, and dilinolein were 17:83, 28:72, and 10:90 in favor of 13-HPODE. The regioisomer ratio with trilinolein (6:94) was higher than the resultant ratio (13:87) when free linoleic acid was used for biotransformations (**Figure 5**).

Stereoselectivity of LOX Isoenzymes with Commercial Substrates. LOXs from plants are reported to catalyze the formation of (*S*)-configured hydroperoxides (*20*), whereas chemical processes such as auto- and photooxidation usually lead to racemates. Stereoisomer ratios of 9- and 13-HPODE from the reactions of LOX1 and LOX2 with all substrates were determined. The regioisomers 9- and 13-HPODE were separated as reduced methyl esters (HODE-Me) on a semipreparative SP-HPLC. The purified regioisomers were analyzed by means of chiral HPLC. The HODE stereoisomers (**Figures 6** and **7**) were determined including autoxidation-derived HODE.

LOX1. The enantiomeric excesses of 9-HPODE catalyzed by LOX1 (**Figure 6**) were determined as follows: Free linoleic acid [ee (%) = 86(*S*)], methyllinoleate [ee (%) = 78(*S*)], monolinolein [ee (%) = 74(*S*)], and PamLinGroPCho [ee (%) = 84(*S*)], confirming the stereoselective formation of 9-HPODE by this enzyme. 9-HPODE derived from dilinolein and trilinolein was analyzed with ee (%) = 6(*S*) and ee (%) = 10(*R*), respectively, indicating the influence of chemical oxidation. 13-HPODE resulting from LOX1 catalysis exhibited an excess of the (*S*)-enantiomer for all esterified substrates. The enantiomeric excesses of 13-HPODE were analyzed for monolinolein [ee (%) = 52(*S*)] and PamLinGroPCho [ee (%) = 50(*S*)], respectively. 13-HPODEs from Me-Lin, dilinolein, and trilinolein were ee (%) = 4(*S*), 4(*S*), and 10(*S*) whereas free linoleic acid was transformed to 13-HPODE with ee (%) = 4(*R*).

LOX2. The isoenzyme LOX2 led to (*S*)-configured 13-HPODE for all substrates (**Figure 7**). High (*S*)-enantiomer excesses were obtained with free linoleic acid [ee (%) = 79-(*S*)], Me-Lin [ee (%) = 78(*S*)], and PamLinGroPCho [ee (%) = 86(*S*)] whereas monolinolein, dilinolein, and trilinolein were catalyzed with enantiomeric excesses of [ee (%) = 70(*S*)], [ee-(%) = 68(*S*)], and [ee(%) = 64(*S*)]. Catalysis of LOX2 yielded 9-HPODE with an excess of the (*R*)-enantiomer for all substrates except for monolinolein [ee (%) = 4(*S*)], which is close to the

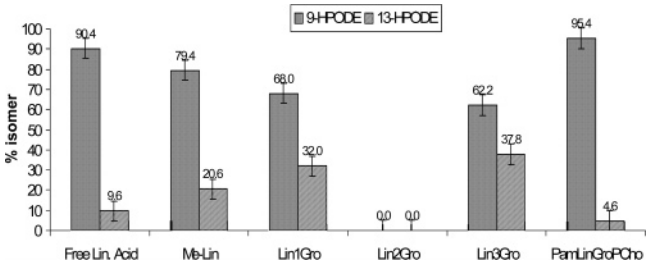

Figure 4. HPODE regioisomer ratios obtained after 2 h of reaction of lipid substrates at pH 6.5 with isoelectric-focused LOX1 enzyme. The confidence interval was calculated at a 95% level with 5 degrees of freedom ($n = 2$, $t = 2.571$).

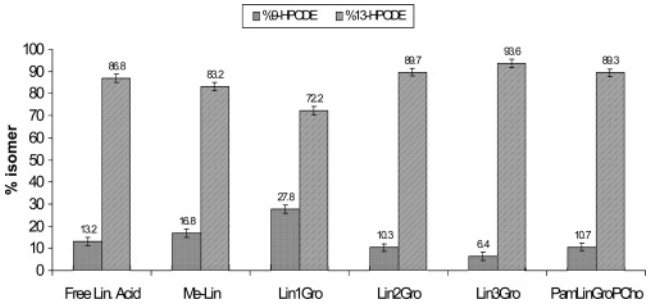

Figure 5. HPODE regioisomer ratios obtained after 2 h of reaction of lipid substrates at pH 6.5 with isoelectric-focused LOX2. The confidence interval was calculated at a 95% level with 5 degrees of freedom ($n = 2$, $t = 2.571$).

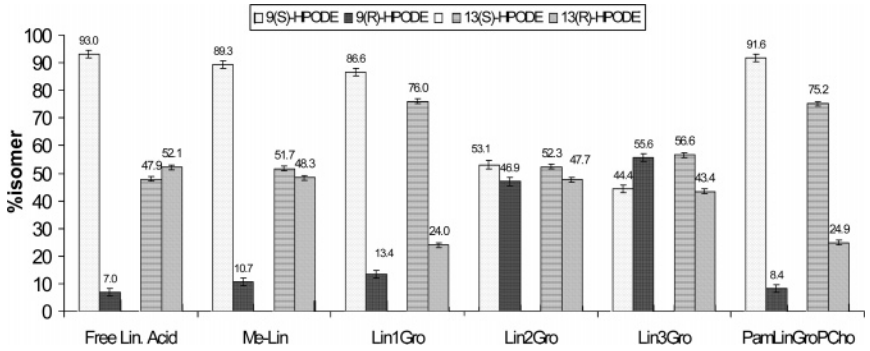

Figure 6. Stereoisomer ratios resulted from the reaction of purified LOX1 isoenzyme (IEF) with lipid substrates. The confidence interval was calculated at a 95% level with 5 degrees of freedom ($n = 2$, $t = 2.571$).

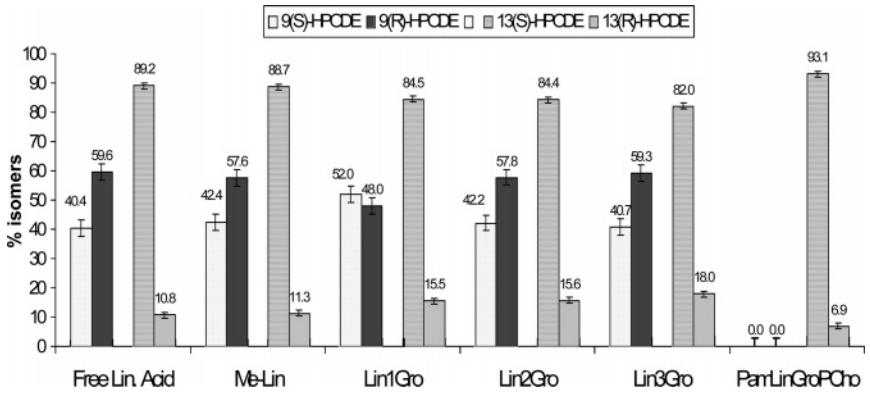

Figure 7. Stereoisomer ratios resulted from the reaction of purified LOX2 isoenzyme by IEF with lipid substrates. The confidence interval was calculated at a 95% level with 5 degrees of freedom ($n = 2$, $t = 2.571$).

racemate. Enantiomeric excesses of 9-HPODE with free linoleic acid, methyl linoelate, dilinolein, and trilinolein were [ee(%) = 19(*R*)], [ee(%) = 16(*R*)], [ee(%) = 16(*R*)], and [ee(%) = 18(*R*)].

Fatty Acid Composition of Extracted Polar and Nonpolar Lipids from Malt. In cereals, lipids are stored in oil droplets or spherosomes containing predominantly triacylglycerols and small amounts of other neutral lipids. The spherosomes are bound to a membrane, which contains polar lipids, predominantly phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) (*30*).

The fatty acid composition of polar and nonpolar lipids extracted from malt is shown in **Table 1**. Explicit differences were analyzed in the two lipid classes, especially related to palmitic and linoleic acid. The relative amount of palmitic acid was 37% in polar and 18% in nonpolar lipids whereas linoleic acid was found with 45 and 63%, respectively. According to Morrison et al. (*30*), the fatty acid composition of malt polar lipids is 1 (14:0), 38 (16:0), 3 (18:0), 9 (18:1), 46 (18:2), and 3% (18:3). Nonpolar lipids were detected by Zürcher (*31*) in malt, and fatty acid compositions for triacylglycerides (neutral lipids) of 23 (16:0), 2 (18:0), 7 (18:1), 61 (18:2), and 7% (18:3) were found.

Regioselectivity of LOX Isoenzymes with Extracted Malt Lipids. The HPODE regioisomer ratios of extracted polar and nonpolar lipids from malt biotransformations are shown in **Figures 8** and **9**. The calculated HODE regioisomer ratios represent the 9-/13-HPODE ratios formed by LOX isoenzymes. Chemical autoxidation occurring during the incubation was estimated by use of blank samples, and these amounts of HODE were subtracted from total HODE. The nonpolar malt lipids were purified by LC to eliminate oxidized lipids whereas the extracted polar lipids were used without further chromatographic purification.

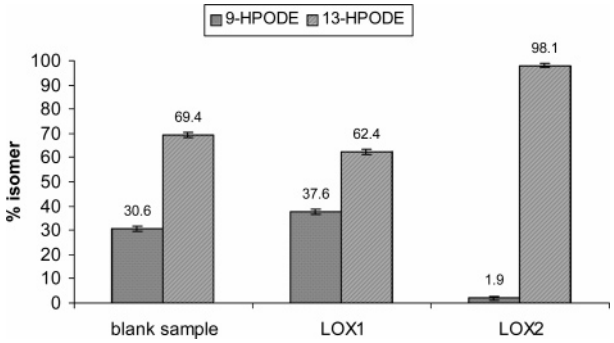

Figure 8. HPODE regioisomer ratios obtained after 2 h of reaction of EPL with IEF-separated LOX. The confidence interval was calculated at a 95% level with 3 degrees of freedom ($n = 2$, $t = 3.182$).

Polar Lipids. The blank sample of extracted polar esterified lipids (**Figure 8**) showed a 9-:13-HPODE ratio of 31:69, which could be explained by an existence of oxygenated lipids in the substrate, which was not purified, and further autoxidation within the oxygen incubation. Comparisons of HPODE amount and ratio from extracted polar lipids and its blank sample after autoxidation were identical within the error margin; therefore, autoxidation did not contribute. The results from LOX biotransformations with extracted polar lipids are weakly interfered by HPODE present in the substrate. The 9-:13-HPODE ratios of extracted polar lipids after 2 h of enzymatic reaction by LOX1 and LOX2 were 38:62 and 2:98, respectively.

Nonpolar Lipids. The blank sample reaction of nonpolar esterified lipids (**Figure 9**) showed a 9-:13-HPODE ratio of 46:54 indicating a substrate autoxidation within the 2 h reaction. The detected hydroperoxide ratio (9-:13-HPODE) after LOX1 and LOX 2 biotransformation was 47:53 and 7:93, respectively.

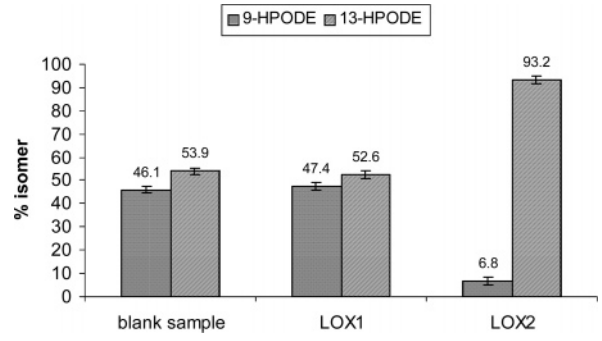

Figure 9. HPODE regioisomer ratios obtained after 2 h of reaction of extracted nonpolar lipids from malt with IEF-separated LOX. The confidence interval was calculated at a 95% level with 3 degrees of freedom ($n = 2$, $t = 3.182$).

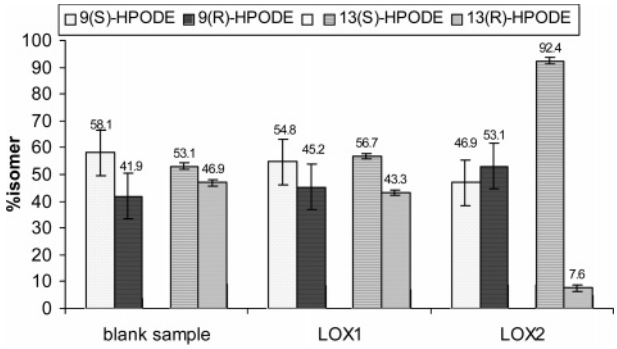

Figure 10. Stereoisomer ratios resulted from biotransformations of IEF-purified LOX isoenzymes with extracted polar malt lipids. The confidence interval was calculated at a 95% level with 3 degrees of freedom ($n = 2$, $t = 3.182$).

Stereoselectivity of LOX Isoenzymes with Extracted Malt Lipids. *Polar Lipids.* The HPODE stereoisomers from LOX catalysis with extracted polar malt lipids were analyzed including malt HODE as well as autoxidation during oxygen incubation. The blank sample of extracted polar malt lipids after 2 h of incubation (**Figure 10**) showed a *S*:*R* ratio of 58:42 and 53:47 for 9-HPODE and 13-HPODE, respectively. **LOX1.** After incubation with LOX1, small enantiomeric changes were detected and a *S*:*R* ratio of 55:45 for 9-HPODE was found whereas the ratio for 13-HPODE was 57:43. Even these ratios were close to a racemic mixture, and to the error margin, all analyzed samples exhibited (*S*)-configuration. **LOX2.** The purified LOX2 isoenzyme transformed the extracted polar lipids to 9-HPODE with *S*:*R* 47:53 indicating a low (*R*)-enantiomer excess whereas 13-HPODE was analyzed with 92:8. The high (*S*)-enantiomeric excess of 13-HPODE [ee (%) = 84(*S*)] points to an enzymatic formation of 13-HPODE by LOX2.

Nonpolar Lipids. HPODE stereoisomers from extracted nonpolar lipids were analyzed including the HPODE resulting from autoxidation during the incubation. The blank sample of extracted nonpolar lipids purified by silica gel column chromatography showed a *S*:*R* ratio of 52:48 for 9-HPODE whereas for 13-HPODE a 53:47 ratio was analyzed. Small increases of (*S*)-enantiomers were detected in the LOX1-incubated sample resulting in a *S*:*R* ratio of 56:44 for 9-HPODE and for 13-HPODE of 55:45. LOX2 catalyzed the formation of (*R*)-9-HPODE from nonpolar lipids in a *S*:*R* ratio of 40:60 whereas for 13-HPODE a *S*:*R* ratio of 91:9 was analyzed confirming the enzymatic formation of 13-HPODE by LOX2.

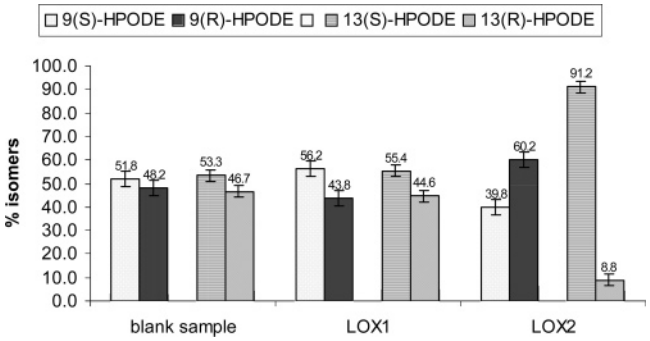

Figure 11. Stereoisomer ratios resulted from biotransformations of IEF-purified LOX isoenzymes with extracted nonpolar malt lipids. The confidence interval was calculated at a 95% level with 3 degrees of freedom ($n = 2$, $t = 3.182$).

DISCUSSION

The application of IEF-separated and partially purified LOX isoenzymes from green malt lacking HPME and lipase activities combined with the estimation of autoxidation processes during incubation represents an important contribution to study the very first step of the LOX cascade of esterified fatty acids. HPODEs were reduced to HODE with subsequent characterization of regio- and stereoisomers. According to the literature, the resulting HPODE can be metabolized by other enzymes such as peroxygenase (*9*), hydroperoxide lyase (*32*), AOS (*1*), and divinyl ether synthase (*33*).

Statistical Analysis. The reproducibility of the analytical method and of the biotransformations was tested by duplicate determinations of each experiment, and analysis and the averages of the results are shown in **Figures 1**−**11**. The confidence intervals of all quantitative data presented (mg, %) were calculated at a 95% confidence coefficient (P = 95%). The coefficient of variation for the GC analysis was lower than 7% whereas for the HPLC analysis it was lower than 3%. The coefficient of variation for the enzymatic reactions (biotransformations) was lower than 3%. Quantification of 9- and 13-HPODE by GC-MS was performed by analyzing the TMS ethers of methyl 9- and 13-hydroxyoctadecanoates corresponding to the TMS ether of methyl 12-hydroxyoctadecanoate as an internal standard. This method was validated by mixing and analyzing authentic standards.

Biotransformation of Esterified Linoleic Acids with LOX1. The experiments carried out with LOX1 showed that autoxidation plays a significant role in the product ratios depending on the substrate. The HPODE amount in the blank sample caused by autoxidation with methyllinoleate and monolinolein (0.10 and 0.14 mg, respectively) represented only 2 and 7% of the total HPODE detected after their enzymatic transformation (**Figure 1**). Nevertheless, the HPODE caused by autoxidation of dilinolein and trilinolein represented 100 and 70%, respectively, of the total concentration (**Figure 1**). The relative activity of LOX1 with the substrates dilinolein and trilinolein as compared to free linoleic acid (**Figure 3**) showed that almost no biotransformation occurred with dilinolein and trilinolein by this enzyme (0 and 0.9% relative activity). However, significant metabolizing capacity was shown with monolinolein (11%), which could represent the participation of LOX1 in the esterified fatty acid metabolism after partial lipase action toward triacylglycerides in green malt. The fact that 70% of the fatty acids of barley and malt lipids are triglycerides (*16*) accounts for the importance of this lipid class in the study of the LOX pathway. LOX1 transformed trilinolein in a 9-:13-HPODE ratio of

62:38 representing a lower regioselectivity than for free linoleic acid (90:10) (**Figure 4**). The *S*:*R* ratio of 9-HPODE (44:56) did not confirm the expected (*S*)-stereoselectivity of LOX1 with trilinolein (**Figure 6**). The results of LOX1 catalysis with trilinolein as substrate were doubtful due to the low amount of enzymatically formed product and also due to the high autoxidation level detected in the blank samples.

Although a low HPODE amount (0.06 mg) was detected after enzymatic transformation of PamLinGroPCho by LOX1 (**Figure 1**), the autoxidation represented only 17% of the total HPODE amount (0.01 mg). The high regioselectivity (9-:13-HPODE = 95:5; **Figure 4**) and the high stereoselectivity (*S*:*R* from 9-HPODE = 92:8; **Figure 6**) detected after the biotransformation confirmed the enzymatic formation of 9-HPODE by LOX1. In comparison with other substrates, it could be assumed that PamLinGroPCho might fit best into the enzyme pocket for lipoxygenation even if the transformation rate is low. Hübke et al. (*20*) reported an increase of (*S*)-enantiomers in a ratio 9-:13-HPODE of 40:60 in polar lipids during 6 days of germination. This result is not in accordance with the reported regioselectivity of LOX2 with PamLinGroPCho as the substrate [9-:13-HPODE = 89:11 (**Figure 5**)]; therefore an unknown (*S*)-9-LOX activity was postulated with polar lipids as substrate.

Biotransformation of Esterified Linoleic Acids with LOX2. The high HPODE amount catalyzed by LOX2 with all esterified derivatives of linoleic acid (Figure 2) reflects a minor problem of the autoxidation. The high regioselectivity (9-:13-HPODE = 6:94) toward trilinolein (**Figure 5**) and the *S*:*R* ratio of 82:18 for 13-HPODE (**Figure 7**) revealed the enzymatic formation of this hydroperoxide by LOX2. Increasing amounts of 13-(*S*)-HODE were analyzed in the neutral storage lipids (component of the lipid bodies) during the germination of cucumber seeds and also in the monolayer of phospholipids surrounding lipid bodies (*10*). A significant increase in the cytosolic concentration of 13-HODE occurred in contrast to linoleic acid showing a preferential release of hydroxylinoleic acid from the lipid bodies (*10*). In *Ricinus* seeds, an enzyme has been detected that specially cleaves hydroxyoleic acid from ester lipids resources (*34*). These data show that an involvement of LOX2 in the lipid mobilization during germination is most likely.

Hübke et al. (*20*) showed a HODE increase with a ratio 9-:13-HODE of 16:84 in triacylglycerol-esterified HODE in 6 days green malt. LOX2 exhibited a 9-:13-HPODE regioselective ratio of 6:94 when trilinolein was used as the substrate (**Figure 5**), elucidating a minor difference with the ratio in vivo. The possible HPODE metabolism performed by peroxidases and other HPME as well as the existence of autoxidation during the 6 days of germination could be responsible for this difference.

9-HPODE represented 28, 10, and 6% of the total enzymatic-catalyzed HPODE with monolinolein, dilinolein, and trilinolein as substrates (**Figure 5**). This amount of 9-HPODE for LOX2 biotransformations must be taken in consideration due to the high enzymatic activity of LOX2 (**Figure 3**) and the high abundance of triacylglycerols in barley and malt lipids. According to Drost et al. (*35*), 13-HPODE is not likely the precursor of *trans*-2-nonenal because its enzymatic cleavage will occur between C_{12} and C_{13} to yield hexanal. Nevertheless, the predictable cleavage of 9-HPODE will result in *cis*-3-nonenal, which can isomerize to *trans*-2-nonenal. Nowadays, attempts to minimize the formation of beer off-flavor precursors are focused on the reduction of LOX1 because mainly 9-HPODE is formed by this enzyme. The presented results reveal the contribution of LOX2 in the formation of 9-HPODE and, consequently, the contribution to the formation of off-flavor products in beer.

The (*R*)-9-HPODE enantiomeric excesses analyzed during LOX2 catalysis with all of the substrates (except monolinolein) (**Figure 7**) indicate this isomer as a substrate in β-oxidation. The β-oxidation activity in germinating barley embryos is proved to be significantly lower than in the aleurone (*36*).

The high regioselectivity (9-:13-HPODE = 11:89) (**Figure 5**) and the high stereoselectivity of 13-HPODE (*S*:*R* = 93:7) (**Figure 7**) detected during transformation of PamLinGroPCho by LOX2 showed that not only nonpolar lipids but also polar lipids are LOX2 substrates. Phospholipids are essential components in the constitution of the oil body membranes of plants (*16*). LOX2 oxidation activity could be the initiating breakdown reaction of these membrane phospholipids and favoring the attack of specific lipases. The membrane rupture would make the lipid body accessible to other enzymes such as LOXs and lipases.

Biotransformation of Extracted Polar Lipids with LOX1 and LOX2. *LOX1*. The catalysis of LOX1 with PamLinGroPCho as substrate (**Figure 4**) resulted in a high regioselectivity (9-:13-HPODE = 95:5). This effect was not found when extracted polar lipids (**Figure 8**) were used as substrate (9-:13-HPODE = 38:62). Although the fatty acid composition of the extracted polar malt lipids (**Table 1**) shows approximately equal amounts of linoleic acid and palmitic acid, 37 and 45%, respectively, as for the substrate PamLinGroPCho, these commercial polar lipids cannot really represent the natural disposition in the seed. According to Parsons and Price (*37*), barley phospholipids contain approximately 51% of PC, 28% of lysophosphatidylcholine, 9% PE, and 2% PI. The amount of HPODE detected in the blank samples (0.17 mg) was 2-fold higher than HPODE (0.08 mg) formed enzymatically by LOX-1 (**Figure 1**). Although the reaction was performed under the same experimental conditions, the autoxidation detected in the blank samples cannot predict exactly the real autoxidation occurring in the enzymatic transformation. Small differences in this prediction could represent a relatively high change in the ratio 9-:13-HPODE detected. The comparison of the stereoisomer ratio (**Figure 10**) of 9-HPODE resulted from the LOX1 (*S*:*R* = 55:45) biotransformation with the *S*:*R* ratio of 9-HPODE from the blank sample (58:42) confirmed that in fact no enzymatic LOX1 catalysis occurred when EPLs were used as the substrate.

LOX2. The relative activity of LOX2 (**Figure 3**) was approximately 6-fold higher than LOX1 (2.3 and 0.4, respectively) toward the polar lipids. The high regioselectivity (9-:13-HPODE = 2:98) (**Figure 8**) and the high stereoselectivity analyzed for 13-HPODE (*S*:*R* = 92:8) (**Figure 10**) combined with the fact that LOX2 synthesis starts at the very first stage of the germination suggest the participation of this enzyme in the oxygenation of polar lipids. This oxygenation of polar lipids would change the structural and functional characteristics of the membranes. This might by initiated by LOX2 attack of triacylglycerols, the main constituents of the spherosomes. Even if the transformation rate of the polar extracted lipids as compared to free linoleic acid is low (2.3%), the detected regio- and stereoselectivity of LOX2 are the highest for this lipid class.

Biotransformation of Extracted Nonpolar Lipids with LOX1 and LOX2. Nonpolar lipids in malt occur predominantly as triacylglycerides (~85%), free fatty acids (~8.5%), diglycerides (~4.5%), and monoglycerides (~1.2%) (*16*). The results shown in **Table 1** indicate that the most common fatty acid in the nonpolar lipid extract is linoleic acid (63%) followed by palmitic acid (18%) and oleic acid (12%).

LOX1. The low amount of HPODE detected after LOX1 biotransformation (0.03 mg) with nonpolar lipids as substrate as compared with the predicted autoxidation (0.04 mg) (**Figure 1**) turns the results doubtful. Although no regioselectivity was detected (9-:13-HPODE = 47:53) (**Figure 9**), a small 9-(*S*)-HPODE increase occurred when LOX1 biotransformation was compared to the blank sample (**Figure 11**). Nevertheless, the very low relative activity presented for LOX1 with nonpolar lipids (0.2%) indicates that probably no enzymatic catalysis was performed by LOX1 with nonpolar lipids.

LOX2. With extracted nonpolar lipids (**Figure 3**), LOX2 showed a high catalysis rate (22.2%). This rate was approximately 4-fold higher than the rate with trilinolein as substrate, which points out the artificial nature of trilinolein. The ratio 9-:13-HPODE (93:7) (**Figure 9**) and the high stereoselectivity shown with 13-HPODE (*S*:*R* = 91:9) (**Figure 11**) reveal the enzymatic formation of 13-HPODE by LOX2 and prove the nonpolar lipids as one natural LOX2 substrate.

LOX2 plays an important role in the formation of HPODE as compared to chemical oxidation. This fact can be confirmed via comparison of HPODE amounts in the blank sample (0.04 mg) with the total amount of HPODE (3.08 mg) catalyzed by LOX2, when nonpolar lipids are used as substrate (Figure 2). The catalysis rate of LOX2 was 77-fold higher than the chemical oxidation rate with nonpolar lipids using the same experimental conditions. As detected by LOX2 with other substrates, the small amount of 6,8% 9-HPODE (**Figure 9**) showed a (*R*)-enantiomer excess ee = 20(*R*) (**Figure 11**). The comparison between the amount of autoxidized 9-HPODE formed (0.02 mg, 50% of 0.04 mg) with the enzymatic formed 9-HPODE (0.21 mg, 6.8% of 3.08 mg) and the resulting *S*:*R* ratio of 40:60 show the biological significance of this hydroperoxide.

Conclusion. The LOX isoenzymes LOX1 and LOX2 from germinated barley (green malt) were purified and successfully separated by IEF. The regio- and stereoselectivity of LOX1 and LOX2 toward free and esterified derivatives of linoleic acid and extracted polar and nonpolar lipids from malt were characterized. LOX1 exhibited a low catalytic activity with complex substrates as compared to free linoleic acid. Residual relative activities lower than 1% with the substrates dilinolein, trilinolein, and PamLinGroPCho and with extracted lipids from malt confirmed this supposition. However, LOX1 catalyzed HPODE formation from PamLinGroPCho with high regioselectivity (9-HPODE:13-HPODE = 95:5) and high (*S*)-9-HPODE stereoseletivity (*S*:*R* = 92:8).

LOX2 catalyzed the formation of HPODE with complex substrates accompanied by a high regio- and stereoselectivity. Trilinolein and extracted polar and nonpolar lipids were oxidized with a ratio 9-:13-HPODE of 6:94, 2:98, and 7:93. The stereoisomer ratios (*S*:*R*) of 13-HPODE with these substrates were 82:18, 92:8, and 91:9 whereas 9-HPODE was analyzed (*R*)-configured with 41:59, 47:53, and 40:60 (*S*:*R*). Although representing less than 10% of the total amount of formed HPODE by LOX2 with trilinolein and extracted polar and nonpolar lipids, 9-HPODE could contribute to the formation of off-flavor in beer since its predictable cleavage is into *cis*-3-nonenal, which can isomerize to *E*-2-nonenal. *E*-2-nonenal is discussed as one flavor active substance in beer, but its contribution in the composition with further ingredients to staling flavor and off-taste is still doubtful (*38*). However, 9-HPODE formed by LOX2 should be taken into consideration as a tentatively off-flavor precursor.

The high regio- and stereoselectivity of LOX2 toward all esterified derivatives of linoleic acid as well as to extracted malt lipids and the fact that LOX2 is just formed during the early stages of the germination suggest that LOX2 possesses different functions than LOX1. LOX2 seems to be involved in the lipid mobilization in germinating barley. This enzyme could be a key enzyme to cause an alteration of the structural and functional characteristics of the polar spherosome membranes. The membrane rupture would make the nonpolar lipid body accessible to other enzymes such as lipases, LOXs, and β-oxidation enzymes. However, the results of the determination of enzyme characteristics and properties by in vitro biotransformations as performed in the present study should only carefully be transferred to their in vivo functions.

With PamLinGroPCho as the substrate, a high regio- and stereoselectivity was detected for both isoenzymes and it could be assumed that this substrate fits best into the enzyme pocket although the rate of enzymatic reaction is low. The enzyme-catalyzed formation of HPODE by LOX2 with extracted nonpolar lipids as substrate was 77-fold higher than the occurred chemical reaction showing clearly that the enzymatic pathway contributes much stronger to HPODE formation than autoxidation. The dual position specificity of LOX1 and LOX2 is dependent on the substrates and on the isoenzyme separation, which was successful by means of IEF. The configuration of the minor HPODE isomers formed by barley LOX isoenzymes was analyzed for the first time.

ABBREVIATIONS USED

HPODE, hydroperoxy octadecadienoic acid; HODE, hydroxy octadecadienoic acid; HPME, HPODE metabolizing enzyme; IEF, isoelectric focusing; Me-Lin, methyl linoleate; Lin1Gro, monolinolein (1-monolinoleoyl-glycerol); Lin2Gro, dilinolein (1,3-dilinoleoyl-glycerol); Lin3Gro, trilinolein (1,2,3-trilinoleoyl glycerol); PamLinGroPCho, 1-palmitoyl-2-linoleoyl-glycero-3-phosphocholine; EPL, extracted polar lipids from malt; ENPL, extracted nonpolar lipids from malt; TMS, trimethylsilyl.

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