Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 29 (2004) 201–209
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Characterization of an enriched lipoxygenase extract from *Aspergillus niger* in terms of specificity and nature of flavor precursors production

Colin E. Hall [a], Florence Husson [b], Selim Kermasha [a,*]

[a] *Department of Food Science and Agricultural Chemistry, McGill University, 21111 Lakeshore, Ste-Anne de Bellevue, Que., Canada H9X 3V9*
[b] *Laboratoire de Microbiologie, ENSBANA, 1 Esplanade Erasme, Campus Universitaire Montmuzard, 21000 Dijon, France*

Received 3 July 2003; received in revised form 29 October 2003; accepted 7 November 2003

Available online 15 April 2004

Abstract

Aspergillus niger was grown for 6 days, and the harvested biomass was homogenized; the resultant supernatant, considered as the crude enzymatic extract, was enriched by ammonium sulfate precipitation. The extract was assayed for its lipoxygenase (LOX) activity using a wide range of polyunsaturated fatty acids (PUFAs), including linoleic, linolenic and arachidonic acids, as substrates. Two pH maxima were determined at 5.0, 10.5. The K_m and V_{max} values indicated that the microbial LOX displayed preferential substrate specificity towards linolenic acid at low pH. The microbial LOX demonstrated preferential substrate specificity towards free fatty acids over the acyl esters of linoleic acid. It was shown that the LOX activity of *A. niger* produced all monohydroperoxy regioisomers of the PUFAs, and there was a predominance of conjugated diene hydroperoxides. Significant production of the unconjugated 10-hydroperoxides of both linoleic and linolenic acids was obtained by the LOX activity. The amounts of 10-hydroperoxides ranged from 15 to 21% of total produced isomers, for linolenic and linoleic acids, respectively. The greatest proportion of the 10-regioisomer was attributed to the maximum activity at pH 5.0. Four major hydroperoxy-eicosatetraenoic acid (HPETE) regioisomers were isolated from the bioconversion of arachidonic acid, including the 8-, 9-, 12- and 15-HPETE, which accounted for approximately 97% of total isomers.

Keywords: *Aspergillus niger*; Lipoxygenase; Polyunsaturated fatty acids; Hydroperoxides; Flavor precursors

1. Introduction

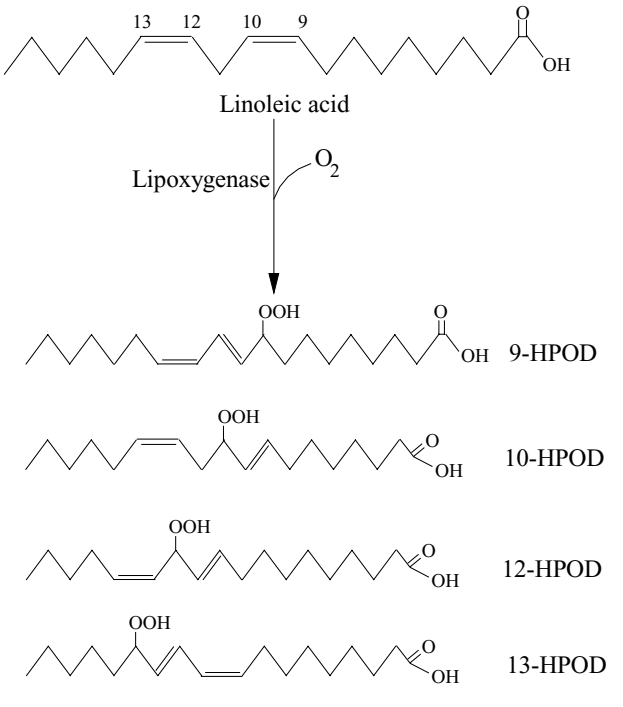
Lipoxygenase (LOX; EC 1.13.11.12) is distributed widely in nature and is responsible for the enzymatic conversion of 1(*Z*),4(*Z*)-pentadiene containing polyunsaturated fatty acids (PUFAs) into stereospecific hydroperoxide isomers, by antarfacial insertion of molecular oxygen at the methylene carbon [1]. Although plant LOXs have been studied widely [2], there has been a paucity of studies investigating the role of these dioxygenases in microorganisms [3]. However, selected microorganisms have been investigated, including bacterial species [4,5], algae [6–8] and industrially important yeasts such as *Saccharomyces cerevisiae* [9].

LOX has been implicated in the biogeneration of both desirable and undesirable volatile flavor compounds in foods, stemming from an initial oxidation of PUFAs that resulted in the formation of PUFA hydroperoxides of linoleic (HPODs) (see Fig. 1), linolenic (HPOTs) and arachidonic (HPETEs) acids [10]. These hydroperoxides have long been considered flavor precursors, and subsequent enzymatic activities such as hydroperoxide lyase (HPL) have been shown to convert them into flavor compounds [10–15].

The present work is part of ongoing research in our laboratory aimed at the biotechnological applications of microbial enzymes in the biogeneration of natural flavors from lipid sources [6,7,9,14–18]. The specific objectives of this study were to recover and to characterize LOX in the fungus *Aspergillus niger*, in terms of pH maxima, substrate specificity and other kinetic parameters as well as the structural characterization of end products, considered to be flavor precursors.

* Corresponding author. Tel.: +1-514-398-7922; fax: +1-514-398-8132.
E-mail address: selim.kermasha@mcgill.ca (S. Kermasha).

1381-1177/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2003.11.013



Hydroperoxide regio-isomers of linoleic acid (HPODs)

Fig. 1. Bioconversion of linoleic acid into the 9-, 10-, 12- and 13-hydroperoxide (HPOD) regioisomers, catalyzed by lipoxygenase.

2. Materials and methods

2.1. Culture growth and harvesting conditions

The growing medium used for *A. niger* contained glucose (50.0 g/l), NH_4NO_3 (3.0 g/l), K_2HPO_4 (2.0 g/l), $MgSO_4 \cdot 7H_2O$ (0.5 g/l), $ZnSO_4 \cdot 7H_2O$ (16.0 mg/l), $FeSO_4 \cdot 7H_2O$ (12.0 mg/l), $CaCl_2 \cdot 4H_2O$ (10.0 mg/l), $MnCl_2 \cdot H_2O$ (5.0 mg/l) and $CuSO_4 \cdot 5H_2O$ (2.0 mg/l) according to the procedure outlined by Kermasha et al. [19]. *A. niger* was induced to sporulate and the resultant spore suspension was counted using a Neubauer Counting Chamber (Hausser Scientific, Horsham, PA), according to the procedure outlined by Perraud et al. [18]. The cultures of *A. niger* were incubated at 27 °C using an orbital shaker at 100 rpm, the glucose consumption was followed up. After harvesting, the biomass was filtered, lyophilized and stored in a −80 °C.

2.2. Preparation and enrichment of enzymatic extract

The lyophilized biomass was homogenized using 0.45–0.50 mm diameter glass beads in an MSK cell homogenizer (Braun, Melsungen, Germany) for 2× 2 min. The resultant suspension was centrifuged (12,000 × *g*, 15 min) and the supernatant was subjected to lyophilization using a FreezeZone® stoppering tray dryer (Labconco, Kansas City, MS). The lyophilized LOX was defatted and subjected to DNA removal as described by Bisakowski et al. [16]; the resultant defatted microbial extract was considered as the crude LOX enzymatic extract (FI). All subsequent steps were performed at 4 °C, unless otherwise indicated.

The enrichment of the crude enzymatic extract was performed with ammonium sulfate precipitation at 0–30, 30–70 and 70–100% of saturation; the precipitated fractions, FIIa, FIIb and FIIc, respectively, were dialyzed and lyophilized as outlined by Bisakowski et al. [16].

2.3. Protein measurement

The protein content of the enzymatic fractions was measured with a modified Lowry method [20], using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as standard for the calibration curve.

2.4. Substrate preparation

The substrates used throughout this study, included linoleic acid (*cis*-9-, *cis*-12-octadecadienoic acid), linolenic acid (*cis*-9-, *cis*-12-, *cis*-15-octadecatrienoic acid), arachidonic acid (*cis*-5-, *cis*-8-, *cis*-11-, *cis*-14-eicosatetraenoic acid), monolinolein (1-mono[(*cis*,*cis*)-9,12-octadecadienoyl]-*rac*-glycerol), dilinolein (1,3-di[(*cis*,*cis*)-9,12-octadecadienoyl]-*rac*-glycerol) and trilinolein (1,2,3-tri[(*cis*,*cis*,*cis*)-9,12-octadecadienoyl]-*rac*-glycerol), were purchased from Nu-Chek-Prep Inc. (Elysian, MN). The substrate stock solutions were prepared at a concentration of 7.5×10^{-3} M in the appropriate buffer solutions (0.1 M) according to the procedure outlined by Perraud et al. [18].

2.5. Enzyme assay

LOX activity was assayed spectrophotometrically using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc., San Raman, CA) according to the procedure outlined by Perraud and Kermasha [14]. The specific activity was defined as micromole of conjugated diene hydroperoxide produced per milligram of protein per minute, using a molar extinction coefficient of 25,000 M^{-1} cm^{-1} [21]. LOX activity was also assayed polarographically, using a Clark oxygen electrode (YSI, Yellow Springs, OH) and a Gilson 5/6 polarograph (Gilson Medical Electronics Inc., Middleton, WI) according to the procedure outlined by Grossman and Zakut [22].

2.6. Effect of pH

The effect of pH on LOX activity was investigated using a wide range of pH buffers solutions (0.1 M), including citrate phosphate for the pH of 4.0–5.5, sodium phosphate for the pH of 6.0–8.0, glycine–NaOH for the pH of 8.5–10.5, phosphate–NaOH for the pH of 11.0–11.5 and KCl–NaOH for the pH of 12.0–13.0.

2.7. Production of hydroperoxide standards

The different PUFA hydroperoxide standards were obtained using selected endogenous LOXs. The 9-HPOD and 9-HPOT were produced by tomato LOX according to the procedures described by Matthew et al. [23]. The 5-HPETE was produced by tomato LOX according to the procedure outlined by Regdel et al. [24]. The 13-HPOD, 13-HPOT and 15-HPETE were produced by soybean LOX type-1B, according to the procedure reported by Hamberg and Samuelsson [25].

2.8. Production and recovery of hydroperoxide end products

The enriched *A. niger* LOX extract FIIb (10 mg protein) was incubated with 30 ml of the substrate (7.5×10^{-3} M), prepared in buffer solutions (0.1 M) of citrate phosphate (pH 5.5) and glycine–NaOH (pH 10.5), according to the method outlined by Perraud and Kermasha [14]. Solid phase extraction (SPE) (Supelclean^{TM} LC-Si 6 ml, Supelco Inc., Bellefonte, PA) was used to purify the hydroperoxides prior to normal-phase high-pressure liquid chromatography (NP-HPLC) according to the procedure outlined by Toshi et al. [26].

2.9. High-pressure liquid chromatography of hydroperoxide end products

The HPODs, HPOTs and HPETEs, obtained from the enzymatic oxidation of the selected PUFA substrates, were reduced to HODs, HOTs and HETEs, respectively, using $NaBH_4$ according to the procedure described by Bisakowski et al. [7]. The hydroxide regioisomers were separated by HPLC using a Beckman Gold system (Beckman Instruments, Fullerton, CA) and an NP-HPLC alphabond silica column (300 mm × 3.9 mm i.d., 5 μm; Alltech Associates Inc., Deerfield, IL), using an ultra-violet diode array detector (UV-DAD) (Beckman, model 168), and an evaporative laser light scattering detector (ELSD) (Varex Corporation, Burtonsville, MD), according to the procedure reported by Perraud and Kermasha [14]. The hydroxides were subjected to gas–liquid chromatography/mass spectrometry (GC/MS) analyses.

2.10. Gas–liquid chromatography/mass spectrometry of hydroperoxide end products

The HODs and HOTs were derivatized into their corresponding methyl trimethylsilyloxystearate (MTMS stearate) derivatives, whereas the HETEs were derivatized into their methyl trimethylsilyloxyarachidate (MTMS arachidate) derivatives, according to the method described by Bisakowski et al. [7]. The derivatized hydroxides were analyzed on HP 6890 Series GC System (Hewlett-Packard Co., Palo Alto, CA), with computerized integration and data handling, and a 5973 Mass Selective Detector (Hewlett-Packard), as outlined by Perraud and Kermasha [14]. Manual tuning of the mass spectrometer was accomplished with the use of perfluorotributylamine (PFTBA) calibrant, while selecting ion *m/z* ratios of 100, 264, and 414 for improved resolution.

3. Results and discussion

3.1. Culture growth of A. niger

The growth cycle of *A. niger* was monitored in terms of changes in dry weight biomass, glucose consumption, pH values and LOX activity. Fig. 2 shows that the biomass for *A. niger* increased rapidly, 0.1–1.75 g dry weight/l, from 2 to 5 days, respectively, reaching a maximal mycelial biomass (1.79 g dry weight/l) and LOX activity (0.038 μmol diene/mg protein min) after 6 days of growth. These findings are in agreement with those reported for

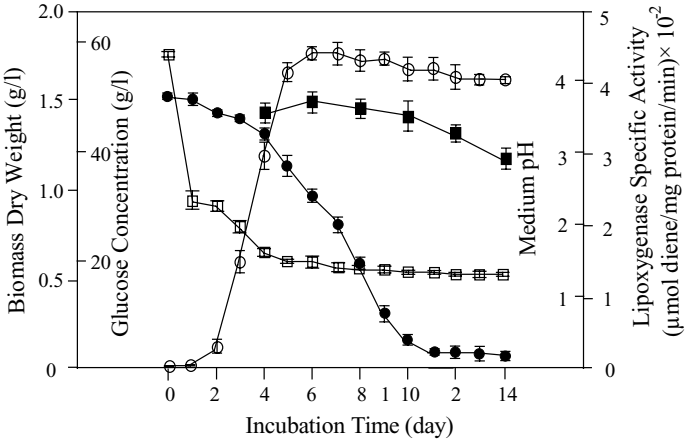


Fig. 2. Changes in glucose concentration (●), media pH (□), biomass dry weight (○) and lipoxygenase specific activity (■) expressed in enzyme units (EU), where 1 EU = 1 μmol diene produced/mg protein min, during a 14-day incubation period for *A. niger*.

Table 1
Partial purification scheme for the lipoxygenase extract from *A. niger*

Enzymatic fraction	Total protein (mg)[a]	Specific activity[b]	Total activity[c]	Recovery[d] (%)	Purification[e] (fold)
Crude (FI)	1125	3.8	42.75	100	1.0
Ammonium sulfate precipitation (FII) at.% of saturation					
0–30% (FIIa)	25	1.1	0.28	1	0.3
30–70% (FIIb)	560	8.4	47.15	110	2.2
70–100% (FIIc)	114	4.4	5.03	11	1.1

[a] Protein concentration was determined as described by Hartree [20].
[b] Specific activity was defined in units of enzyme = (μmol conjugated diene hydroperoxides/mg protein min) × 10^{-2}.
[c] Total was defined as μmol conjugated diene hydroperoxides/min.
[d] Recovery percentage was defined as the ratio of the total activity of an enzymatic fraction to that of the crude, multiplied by 100.
[e] Purification fold was defined as the specific activity of an enzymatic fraction divided by the specific activity of the crude.

Penicillium camemberti by Perraud and Kermasha [14]. Available glucose decreased from 50 to 40 g/l after 5 days of growth; however, the slow decline of glucose concentration is in contrast to that reported for *Geotrichum candidum* cultures, where complete utilization of glucose was observed after 5 days of incubation [18]. A rapid drop in *A. niger*'s medium pH, from 4.5 to 2.2, resulting from the accumulation organic acids [27], was evident after 24 h of incubation, with a slight steady decline over the following 13 days of the growth period to pH 1.4.

3.2. Enrichment of LOX enzymatic extract

Table 1 summarizes the enrichment of the crude LOX enzymatic fraction (FI) from *A. niger*, obtained by ammonium sulfate precipitation at 0–30% (FIIa), 30–70% (FIIb) and 70–100% (FIIc). Fraction FIIb yielded the highest specific activity (0.084 μmol diene/mg protein min) and a purification fold of 2.2; this fraction was considered as the enriched LOX enzymatic extract and used for subsequent studies.

Enrichment of LOXs by ammonium sulfate fractionation of microbial enzymatic extracts were also reported for *Fusarium oxysporum* [16], *Chlorella pyrenoidosa* [17] and *Saccharomyces cerivisiae* [6], with purification folds of 13.3, 51.8 and 3.2, respectively. Purification folds of 2.0 and 2.7 for the LOX extracts from *P. camemberti* [14] and *G. candidum* [18], respectively, are close to that (2.2) determined for *A. niger*.

3.3. Effect of pH on enriched LOX activity

The maximum pH for the LOX activity was investigated, using linoleic acid as substrate. The results (Fig. 3) demonstrate the presence of two pH maxima for the LOX activity of the enriched extract from *A. niger*, 5.0–10.5. However, the rate of enzymatic oxidation was determined to be greater when measured by the polarographic method as opposed to that measured spectrophotometrically. A similar trend was observed with the LOX of *G. candidum* [18], where both spectrophotometric and polarographic methods were employed. The difference in relative higher enzyme activity shown by the polarographic method may be attributed to the nonselective consumption of oxygen by other enzymes present in the reaction homogenate, whereas the spectrophotometric monitoring at 234 nm yields information specific to the production of conjugated diene moieties [22]. Kristie

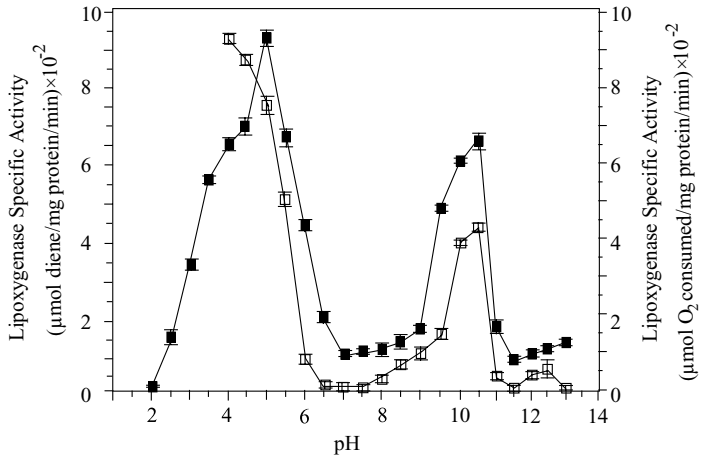


Fig. 3. Effect of pH on the specific activity of an enriched lipoxygenase extract from *A. niger* using linoleic acid as substrate expressed in enzyme units (EU), where 1 EU = 1 μmol diene produced/mg protein min and 1 μmol O_2 consumed/mg protein min, determined using spectrophotometry at 234 nm (□) and polarography (■), respectively.

and Thompson [28] reported that a variety of substances in the crude extract can interfere with spectrophotometric absorbance, and that polarographic methods can be very effective for purified enzymes.

The presence of both acidic and basic pH maxima for microbial LOXs was also reported in the literature. Perraud et al. [18] determined that the LOX activity of *G. candidum* displayed two pH maxima, 3.75 and 8.0. The LOX of the alga *C. pyrenoidosa* [6] was found to possess a maximum of activity at pH 4.5, whereas the bacterial LOX from *Thermoactinomyces vulgaris* possessed a single maximum at 6.5 [5].

3.4. Kinetic studies of the enriched LOX

K_m and V_{max} values for the enriched LOX extract from *A. niger* (Table 2), using linoleic and linolenic acids as substrates, were calculated from Lineweaver–Burk plots. The assays at pH 5.0 were conducted spectrophotometrically using 7.5×10^{-4} M substrate solution, whereas those at pH 10.5 were conducted polarographically using 7.5×10^{-3} M, according to the procedure outlined by Perraud et al. [18].

Using linoleic acid as substrate, the K_m values for the enriched LOX were determined to be 0.092 and 1.27 mM at pHs 5.0 and 10.5, respectively, whereas the V_{max} values indicated a higher rate of oxidation for pH 5.0 than that for 10.5, 0.078 and 0.067 μmol diene/mg protein min, respectively. The catalytic efficiency value, calculated as the ratio of V_{max} to K_m, at pH 5.0 (848), was 16-fold greater than that at pH 10.5 (53). Perraud et al. [18] reported a 16-fold catalytic activity for the LOX of *G. candidum* at pH 3.75 (1051) compared to that at 8.0 (54).

Using linolenic acid as substrate, the K_m and V_{max} values for the enriched LOX at pH 5.0 were 0.095 mM and 0.084 μmol diene/mg protein min, respectively, with a catalytic efficiency (884) close to that obtained with linoleic acid. K_m values reported for the LOXs of *C. pyrenoidosa* [6], *Fusarium proliferatum* [17] and *G. candidum* [18] are close to that of *A. niger*. Values for the V_{max} of microbial LOXs vary widely, including 0.401 μmol diene/mg protein min for *C. pyrenoidosa* [6] and 0.059 μmol diene/mg protein min for *T. vulgaris* [5]. The overall results indicate that the lower K_m and higher V_{max} values were obtained for the enriched LOX at pH 5.0.

3.5. Substrate specificity of LOX using selected PUFAs and fatty acyl esters

Table 2 indicates that the enriched LOX at pH 5.0, demonstrated substrate specificity for linoleic, linolenic and arachidonic acids, of 0.084, 0.086, and 0.072 μmol diene/mg protein min, respectively, whereas that at pH 10.5 was 0.031, 0.026, and 0.028 μmol diene/mg protein min, respectively. Perraud et al. [18] reported that the LOX of *G. candidum* displayed a substrate preference for linolenic acid at pH 3.75 and arachidonic acid at pH 8.0. However, most microbial LOXs showed preferential substrate specificity towards linoleic acid, including *F. oxysporum* [16], *C. pyrenoidosa* [6], *S. cerivisiae* [9], and *P. camemberti* [14].

Table 2 also demonstrates the enriched LOX from *A. niger* showed a preferential substrate specificity towards free fatty acids, including linoleic, linolenic and arachidonic acids with 100, 102 and 85% relative activity, respectively, in comparison to the fatty acid acyl-glycerols, including mono-, di- and trilinolein, with 50, 45 and 39% relative activity, respectively. Bisakowski et al. [17] reported that LOX from *F. proliferatum* demonstrated low substrate specificity towards fatty acid acyl-glycerols, including mono-, di- and trilinolein, with 36.2, 37.8 and 40.7% relative activity, respectively, whereas Perraud and Kermasha [14] indicated the same trend with LOX from *Penicillium roqueforti*, with 14, 17 and 15% relative activity, respectively. However, Feussner and Wasternack [29] reported that LOX from cucumber cotyledon showed a higher substrate specificity towards acyl esters, including trilinolein; these authors suggested that the high substrate specificity might be due to the wide entry afforded by the enzyme's active site.

Table 2
Substrate specificity of the lipoxygenase activity of the enriched extract from *A. niger* (FIIb), obtained by ammonium sulfate precipitation at 30–70% of saturation

Substrate	pH 5.0		pH 10.5	
	Specific activity[a]	Relative activity[b] (%)	Specific activity[a]	Relative activity[b] (%)
Linoleic acid	8.4 (0.4)[c]	100	3.1 (0.21)[c]	100
Linolenic acid	8.6 (0.4)[c]	102	2.6 (0.36)[c]	84
Arachidonic acid	7.2 (0.4)[c]	85	2.8 (0.33)[c]	90
Monolinolein	4.2 (0.1)[c]	50	0.06 (0.01)[c]	2
Dilinolein	3.8 (0.2)[c]	45	n.d.[d]	–
Trilinolein	3.3 (0.2)[c]	39	n.d.[d]	–

[a] Specific activity was defined in units of enzyme = (μmol conjugated diene hydroperodixe/mg protein min) $\times 10^{-2}$ and was determined spectrophotometrically by monitoring the increase in absorbance at 234 nm.
[b] Relative activity was expressed as a percentage with respect to the specific activity, determined for linoleic acid as substrate, at the relevant pH.
[c] Standard deviation of triplicate samplings.
[d] Not detected.

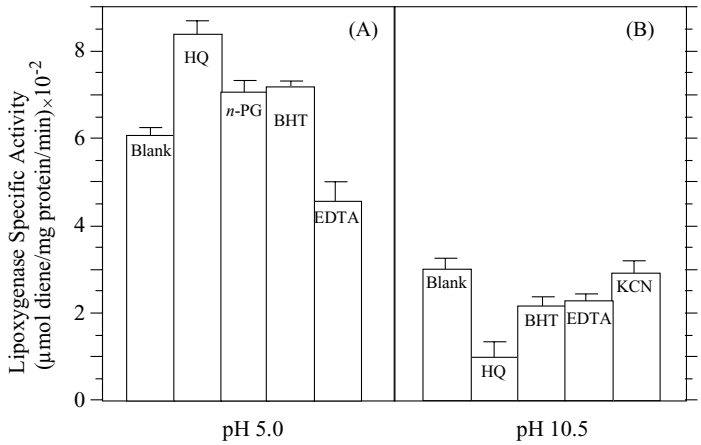


Fig. 4. Effect of selected chemical substances on the specific activity of an enriched lipoxygenase enzymatic extract from *A. niger* at pH (A) 5.0 and (B) 10.5: (blank) without inhibitor, (HQ) 0.25 mM hydroquinone, (*n*-PG) 0.1 mM *n*-propyl-gallate, (BHT) 0.1 mM butylated hydroxytoluene, (EDTA) 5 mM ethylenediaminetetracetate, and (KCN) 100 mM potassium cyanide.

3.6. Effect of selected chemicals on LOX activity

The results (Fig. 4A) indicate that, at pH 5.0, hydroquinone (HQ; 0.25 mM), butylated hydroxy toluene (BHT; 0.1 mM) and *n*-propyl gallate (*n*-PG; 0.1 M) were slightly activating on the LOX activity of the enriched extract, with a 37.7, 16.4 and 19.7% increase in enzymatic activity, respectively; the chemicals, however, are widely believed to be inhibitory to LOX activity [18,28], and displayed inhibition of LOX at pH 10.5. Antioxidants like *n*-PG and BHT reduce the redox iron in LOX from the catalytically active ferric to the inactive ferrous form [17]. Iron ligand inhibitors such as EDTA and KCN chelate ferric and ferrous ions, inhibiting LOX activity [28]. Eskin et al. [30] reported that the use of HQ, BHT and *n*-PG at acidic pH decreases their effectiveness. An increase in activity of LOX from *G. candidum* was reported at pH 3.75 using selected chemicals, including HQ, *n*-PG and BHT [18]. However, at pH 10.5 the inhibition of the enriched LOX activity by HQ and BHT (Fig. 4B) was demonstrated, with a 67.8 and 29.0% decrease in enzyme activity, respectively. In addition, the effect of *n*-PG could not be investigated for the LOX from *A. niger* at pH 10.5, since *n*-PG has been shown to auto-oxidize at pH values greater than 9.0 [28]. Perraud et al. [18] reported that the LOX activity of *G. candidum* at pH 8.0 was inhibited by 72 and 30%, using HQ and *n*-PG, respectively. The most effective inhibitor for LOX activity of *A. niger* at pH 10.5 was HQ (Fig. 4B), which resulted in a decrease of 67.8% in the enzyme activity.

The results (Fig. 4A and B) also indicate that addition of 5 mM of ethylenediaminetetraacetate (EDTA) inhibited LOX activity of the enriched extract 26.2, and 25.8% at pH 5.0 and 10.5, respectively. Perraud et al. [18] reported that using 5 mM of EDTA inhibited the LOX activity from *G. candidum* at pH 3.75 by 14.0%.

The experimental findings (Fig. 4B) indicate that the addition of 100 mM KCN inhibited the enzyme activity of LOX from *A. niger* at pH 10.5 by 9.7%. Perraud et al. [18] reported that the addition of 100 mM KCN inhibited the LOX activity of *G. candidum* at pH 8.0 by 61.0%. Bisakowski et al. [17] indicated that the addition of 80 mM KCN inhibited the LOX activity of *F. proliferatum* by 90.0%.

3.7. Characterization of LOX end products

Fig. 5 shows chromatograms of the NP-HPLC elution profiles of linoleic acid hydroxides (Fig. 5A_1, A'_1, B_1, B'_1, C_1, and C'_1) obtained by the LOX activity of soybean type-1B (A_1), tomato (B_1) and *A. niger* at pH 5.0 (C_1). Characterization of the separated hydroxides was performed with a UV/DAD detector at 234 nm (Fig. 5A_1, B_1 and C_1) and an ELSD detector (Fig. 5A'_1, B'_1 and C'_1). The tentative characterization of HOD, HOT and HETE regioisomers, obtained from the enriched LOX of *A. niger* was based on the retention times and λ_{max} absorbances of the standards and with reference to the literature on tomato LOX [23,24] and on soybean LOX type-1B [11,25].

Using linoleic acid as substrate, peaks *a* and *a′* were characterized as 13-HOD *cis*, *trans* and 13-HOD *trans*, *trans* and co-eluted with peaks 1, 13-HOD *cis*, *trans* and peak 1′, 13-HOD *trans*, *trans*. Peak *b* was identified as the 9-HOD (*cis*, *trans*) and co-eluted with peak 4 produced by *A. niger* (Fig. 5C_1). Peaks 2 and 3 were determined to be the 12- and the 10-HOD, respectively. The NP-HPLC of methyl-linoleate hydroxides [31] and linoleic acid hydroxides [14] have been characterized; according to these authors, the order of NP-HPLC elution of regioisomers was 13-, 12-, 10- and 9-regioisomers, where only the 13- and 9-regioisomers possess an absorbance at 234 nm.

Using linolenic acid as substrate peaks *c* and *c′* were identified as 13-HOT *cis*, *trans* and 13-HOT *trans*, *trans*, respectively (Fig. 5A_2), and co-eluted with peaks 5, and 5′, respectively, obtained by *A. niger* LOX (Fig. 5C_2). Peak *d*, identified as the 9-HOT (*cis*, *trans*), was obtained by tomato

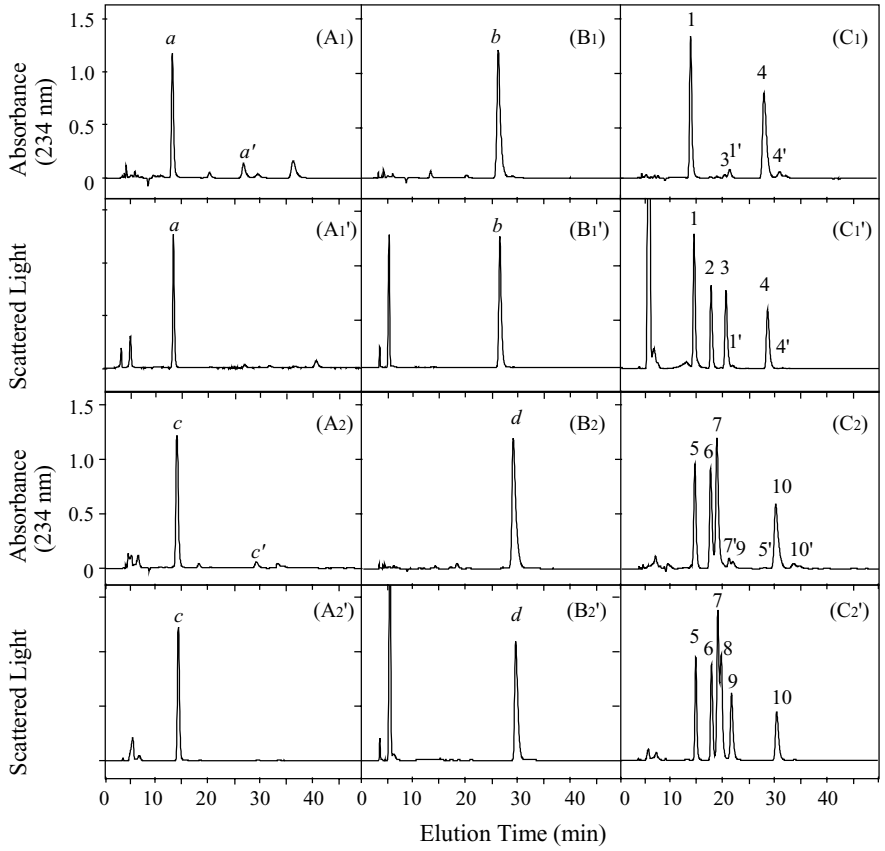


Fig. 5. High-pressure liquid chromatograms of hydroxides of linoleic acid (HODs), using LOX from soybean type-1B (A_1 and A'_1), tomato (B_1 and B'_1) and *A. niger* (C_1 and C'_1), as well as hydroxides of linolenic acid (HOTs), using LOX from soybean type-1B (A_2 and A'_2), tomato (B_2 and B'_2) and *A. niger* (C_2 and C'_2), using ultraviolet diode-array detection at 234 nm (A, B and C) and evaporative laser light scattering detection (A′, B′ and C′).

LOX (Fig. 5B_2) and co-eluted with peak 10, produced by *A. niger* LOX (Fig. 5C_2). The HOTs obtained by *A. niger* LOX produced peaks 5–10′ (Fig. 5C_2). Characterization of peaks 5, 6, 7 and 10 was performed at 234 nm, and after collection and GC/MS analyses, were determined to be the conjugated 13-, 12-, 16- and 9-HOTs of linolenic acid (Fig. 5C_2). The use of ELSD characterized peaks 8 and 9, determined to be the unconjugated 10- and 15-HOTs (Fig. 5C'_2). Spectral scanning of the end products for both linoleic and linolenic acids (not shown), indicated that peaks 1′, 4′, 5′, 7′ and 10′ are characterized as the 13- and 9-HOD and the 13-, 16- and 9-HOT, possessing *trans, trans* geometry and displaying λ_{max} values close to those of other end products reported in the literature [14]. The hydroxide isomers (Fig. 5C'_1 and C'_2) were hypsochromically shifted to a smaller maximal wavelengths, 232 nm, in comparison to the *cis, trans* isomers, characterized at 234 nm [26].

Fragmentation patterns of GC/MS analyses (Fig. 6) of LOX end products were consistent with the presence of the 13-, 12-, 10- and 9-hydroxide regioisomers of linoleic acid [7]. Four major peaks, each consisting of two intense ion abundances of predicted masses, were characterized for their MTMS derivative regioisomers (I, II, III and IV).

The relative quantitative production of HOD, HOT and HETE regioisomers were determined by integrating ion abundances during GC/MS analyses, the results of this integration are shown as percentages of total isomers detected for each of the investigated PUFAs (Table 3).

The major products of the enzymatic oxidation of linoleic acid by the LOX extract from *A. niger* were conjugated diene hydroxides with approximately 60% of 9- and 13-HOD regioisomers, rising to almost 85% at pH 10.5. These results are in agreement with those reported for the bacterial LOX from *T. vulgaris*, which was also noted to catalyze the dioxygenation of linoleic acid into the predominantly conjugated diene hydroperoxide species [4,5].

An appreciable amount of 10-HOD, 21.4% of total hydroxide characterized, was also obtained by the oxidation of linoleic acid by the enriched LOX from *A. niger* at pH 5.0; this value is higher than that obtained by Perraud and Kermasha [14] for *P. camemberti* at its acidic maximum (8%). At higher pH the distribution of unconjugated regioisomers of linoleic acid, decreased to 8.0 and 7.9% for the 10- and 12-regioisomers, respectively. These results suggest that the LOX of *A. niger* was capable of producing adequate unconjugated hydroperoxides to evolve appreciable amounts of volatile compounds when acted upon by HPL

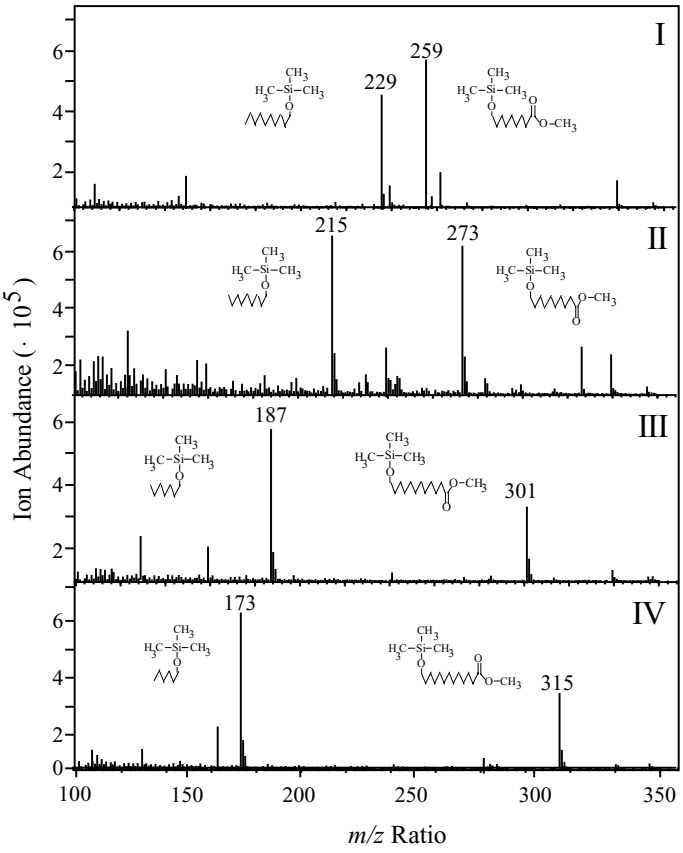


Fig. 6. Mass spectra of the methyl trimethylsilyloxy (MTMS) stearate derivatives of hydroperoxides of linoleic acid produced by the lipoxygenase activity of the *A. niger* enriched enzyme extract: (I) 9-MTMS, (II) 10-MTMS, (III) 12-MTMS and the (IV) 13-MTMS stearate derivatives.

Table 3
Characterization of the relative percentages of hydroperoxide regioisomers, obtained by the enzymatic activity of an enriched LOX extract from *A. niger* at two pH maxima, using linoleic, linolenic and arachidonic acids as substrates

Substrate	Hydroperoxide regioisomer	Hydroperoxide isomers[a] (%)	
		pH 5.0	pH 10.5
Linoleic acid	9-	30.1 (2.1)[b]	44.8 (1.6)[b]
	10-	21.4 (1.3)[b]	8.0 (0.7)[b]
	12-	18.1 (0.6)[b]	7.9 (0.4)[b]
	13-	30.4 (1.8)[b]	39.3 (1.1)[b]
Linolenic acid	9-	11.8 (1.0)[b]	14.3 (1.1)[b]
	10-	15.4 (0.8)[b]	10.4 (0.5)[b]
	12-	18.3 (0.7)[b]	18.9 (0.7)[b]
	13-	19.6 (0.8)[b]	25.7 (1.9)[b]
	15-	11.0 (0.6)[b]	5.5 (0.4)[b]
	16-	23.9 (0.7)[b]	25.2 (1.1)[b]
Arachidonic acid	8-	6.9 (0.3)[b]	n.d.[c]
	9-	38.7 (2.1)[b]	n.d.[c]
	12-	25.0 (0.7)[b]	n.d.[c]
	15-	29.4 (1.3)[b]	n.d.[c]

[a] The hydroperoxide regioisomer was quantified by ion abundances, corresponding to the *m*/*z* ratios of its derivative, detected in GC/MS analyses, and expressed as a percentage relative to all hydroperoxides detected.
[b] Standard deviation of triplicate samplings.
[c] Not determined.

activity. The presence of 10-HPOD has been implicated in the production of volatile eight-carbon compounds, such as 1-octen-3(*R*)-ol [32]. Similarly, the preferential bioconversion of the 10-hydroperoxide of linolenic acid may be an important pathway in the production of other compounds such as 1,5-octadiene-3(*R*)-ol, present in some seafoods and fungi [33,34]. Volatiles detected in the headspace of extracts of *Aspergillus* sp. were shown to possess both 1-octen-3(*R*)-ol and 1,5-octadiene-3(*R*)-ol in organoleptically significant concentrations [35].

GC/MS analyses of end products resulted from the bioconversion of arachidonic acid by the LOX activity of *A. niger* were determined to be predominantly the 8-, 9-, 12- and 15-HPETE. Several studies have determined the oxidation products of bioconversion of arachidonic acid by LOX to be a mixture of HPETE regioisomers. Kuo et al. [8] determined the production of the 15-, 12- and possibly 8-HPETE from algal source LOX. Tomato source LOX has been shown to yield the 5-, 8- and the 11-HPETE, when incubated with arachidonic acid [24]. The LOX of cucumber cotyledons was shown to produce a mixture of 15-, 12- and 8-HPETE [29]. Several authors suggested that these HPETE isomers are “flavor precursors”, thought to be substrates for the production of a variety of melonlike fragrances in algae and fish [36,37].

4. Conclusion

The enriched LOX extract from *A. niger* displayed a wide range of specificity towards free PUFAs, including linoleic, linolenic and arachidonic acids as well as acyl esters of linoleic acid. The enzyme showed two pH maxima. Characterization of the end products, the hydroperoxide regioisomers of PUFAs, confirmed the wide range of selectivity of the LOX from *A. niger*. The overall result suggested that the enriched LOX from *A. niger* showed many of the characteristics of other microbial LOXs in terms of enzyme specificity and nature of flavor precursor end products.

Acknowledgements

This research was supported by a Discovery Grant from the Natural Science and Engineering Research Council of Canada (NSERC) and by the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (CORPAQ).

References

[1] C. Su, E.H. Oliw, J. Biol. Chem. 273 (1998) 13072.
[2] M.A. Fuller, H. Weichert, A.M. Fischer, I. Feussner, H.D. Grimes, Arch. Biochem. Biophys. 388 (2001) 146.
[3] G. Feron, P. Bonnarme, A. Durand, Trends Food Sci. Technol. 7 (1996) 285.
[4] D. Iny, S. Grossman, A. Pinsky, Int. J. Biochem. 25 (1993) 1325.
[5] D. Iny, A. Pinsky, M. Cojocoru, S. Grossman, Int. J. Biochem. 25 (1993) 1313.
[6] B. Bisakowski, S. Kermasha, P. Marsot, Biotechnol. Appl. Biochem. 21 (1995) 39.
[7] B. Bisakowski, X. Perraud, S. Kermasha, Biosci. Biotech. Biochem. 61 (1997) 1262.
[8] J.M. Kuo, A. Hwang, H.H. Hsu, B.S. Pan, J. Agric. Food Chem. 44 (1996) 2073.
[9] B. Bisakowski, S. Kermasha, C. Schuepp, World J. Microb. Biotechnol. 11 (1995) 494.
[10] J.L. Kinderlerner, J. Appl. Bacteriol. Symp. Suppl. 18 (1989) S133.
[11] H. Kühn, R. Wiesner, V.Z. Lankin, A. Nekrasov, L. Alder, T. Schewe, Anal. Biochem. 160 (1987) 24.
[12] D.J. Hawkins, H. Kühn, E.H. Petty, A.R. Brash, Anal. Biochem. 173 (1988) 456.
[13] D. Martini, G. Iacazio, J. Chromatogr. 790 (1997) 235.
[14] X. Perraud, S. Kermasha, J. Am. Oil Chem. Soc. 77 (2000) 335.
[15] S. Kermasha, X. Perraud, B. Bisakowski, F. Husson, J. Mol. Cat. 19/20 (2002) 479.
[16] B. Bisakowski, S. Kermasha, L.M. Klopfenstein, Process. Biochem. 30 (1995) 261.
[17] B. Bisakowski, S. Kermasha, V. Lavorel, J.M. Belin, Food Biotechnol. 9 (1995) 189.
[18] X. Perraud, S. Kermasha, B. Bisakowski, Process. Biochem. 34 (1999) 819.
[19] S. Kermasha, F. Pellerin, B. Rovel, M. Goetghebeur, M. Metche, Biosci. Biotech. Biochem. 57 (1993) 1420.
[20] E.P. Hartree, Anal. Biochem. 48 (1972) 422.
[21] P.K. Surrey, Plant Physiol. 39 (1964) 65.
[22] S. Grossman, R. Zakut, Determination of the activity of lipoxygenase (lipoxidase), in: D. Glick (Ed.), Method of Biochemical Analysis, Wiley, New York, 1979, p. 303.
[23] J.A. Matthew, H.W.S. Chan, T.A. Galliard, Lipids 12 (1977) 324.
[24] D. Regdel, H. Kühn, T. Schewe, Biochim. Biophys. Acta 1210 (1994) 297.
[25] M. Hamberg, B. Samuelsson, J. Biol. Chem. 40 (1967) 953.
[26] T.G. Toshi, F. Stante, P. Capella, G. Lercker, J. High Resol. Chromatogr. 18 (1995) 764.
[27] G.J. Tortora, B.R. Funke, C.L. Case, Microbial metabolism, in: D. Fox (Ed.), Microbiology an Introduction, 6th ed., Addison-Wesley/Longman, Menlo Park, CA, 1997, p. 112.
[28] D. Kristie, J.E. Thompson, Phytochemistry 28 (1989) 2577.
[29] I. Feussner, C. Wasternack, Fett Lipid 100 (1998) 146.
[30] N.A.M. Eskin, S. Grossman, A. Pinsky, Crit. Rev. Food Sci. Nutr. 9 (1977) 1.
[31] M.J. Thomas, W.A. Pryor, Lipids 15 (1979) 544.
[32] M. Wurzenberger, W. Grosch, Biochim. Biophys. Acta 795 (1984) 163.
[33] D.B. Josephson, R.C. Lindsay, D.A. Stuiber, J. Agric. Food Chem. 31 (1983) 326.
[34] M. Wurzenberger, W. Grosch, Lipids 21 (1986) 261.
[35] E. Kaminski, S. Stanwicki, E. Wasowicz, Appl. Microbiol. 27 (1974) 1001.
[36] R.J. Hsieh, J.B. German, J.E. Kinsella, J. Agric. Food Chem. 44 (1988) 741.
[37] C.H. Zhang, T. Hirano, T. Suzuki, T. Shirai, Nippon. Suisan Gakkaishi 58 (1992) 559.