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Process Biochemistry 36 (2000) 1–7

PROCESS BIOCHEMISTRY

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Characterization of lipoxygenase activity from a partially purified enzymic extract from *Morchella esculenta*

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Received 22 November 1999; received in revised form 29 February 2000; accepted 3 March 2000

Abstract

A crude extract (FI) from *Morchella esculenta* was partially purified using ammonium sulphate precipitation at 0–60, 60–80 and 80–100% of saturation to obtain fractions (FIIa), (FIIb) and (FIIc), respectively. The highest specific lipoxygenase (LOX) activity was obtained in fraction (FIIa) which showed a recovery of 12.8% and a 2.5-fold increase in purification. Partially purified LOX extract exhibited optimal activity at the acidic pH of 3.0 and showed 62.5% of the maximal activity in the pH range of 4.0–6.0 and less than 12.5% activity in the pH range of 7.0–10.0. Kinetic studies indicated that the LOX activity of fraction (FIIa) had a V_{max} of 0.314 μmol hydroperoxide mg protein^{-1} min^{-1} and a corresponding K_m value of 1.59×10^{-4} M. The enzymic activity exhibited a strong specificity towards linoleic acid as substrate while only 29% activity was observed using linolenic acid and approximately 11% was obtained with mono-, di- and trilinolein; however, LOX activity showed a relatively strong affinity (83%) towards arachidonic acid as substrate. The enzymic activity of fraction (FIIa) catalyzed the bioconversion of linoleic acid at pH 6.0 into the corresponding 9-, 10-, 12- and 13-hydroperoxides at a ratio of 36:24:14:26, respectively.

Keywords: Lipoxygenase; *Morchella*; Partial purification; Characterization; End-products; Flavour precursors

1. Introduction

Lipoxygenase (EC 1.13.11.12) is a dioxygenase that catalyzes as an initial reaction, the hydroperoxidation of linoleic acid and other polyunsaturated fatty acids (PUFAs) containing a *cis*,*cis*-1,4-pentadiene moiety [1]. The type of regio- and stereo-specific hydroperoxides derived from PUFAs by LOX biocatalysis determines the type of volatile compounds produced including short-chain carbonyl compounds and alcohols responsible for the organoleptic properties of food [2]. The presence of LOX activity in microorganisms including *Aspergillus niger*, *Aspergillus flavoryzea*, *Aspergillus fumigatus*, and *Penicillium glaucum* [3] was first reported in the early 1950s. An enzyme preparation from *Bacillus* sp. [4] and *Pseudomonas aeruginosa* [5] possessing LOX activity was later investigated. Since then, LOX activity has been reported in the fungi *Lagenidium giganteum* [6], *Saprolegnia parasitica* [7], *Geotrichum candidum* [8], *Fusarium proliferatum* [9–12] and *Fusarium oxysporum* [13,14]. A fatty acid dioxygenase from the fungus *Gaeumannomyces graminis*, responsible for the bioconversion of linoleic acid into 8-hydroperoxyoctadecadienoic acid (8-HPOD), was also reported [15–18]; a similar activity was present in the fungus *Laetisaria arvalis* [19] and the mould *Aspergillus nidulans* [20]. The presence of a manganese LOX was reported in the filtrate of the culture medium of *G. graminis* [21].

In mushrooms such as *Psalliota bispora*, LOX activity was reported to be responsible for the bioconversion of linoleic acid into 10-HPOD which was in turn enzymically cleaved to produce octenol and oxodecenoic acid [22–26]. The occurrence of LOX activity was also suggested by the production of eight-carbon compounds in the fungi *Penicillium* and *Aspergillus* [27] and edible mushrooms [28] such as *Agaricus bisporus* [29,30], *Agaricus campestris* [31] and *Pleurotus pul-*

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0032-9592/00/$ - see front matter
PII: S0032-9592(00)00159-X

monarius [32–34]. Moreover, similar enzymic activity has been suggested in the production of important flavor compounds in blue cheese by *Penicillium roqueforti* [35] and in Camembert cheese by *Penicillium camemberti* and *Penicillium caseicolum* [36,37].

The present work is part of ongoing research in this laboratory [8–11,14,38,39] and is aimed at investigating the presence of LOX in *Morchella esculenta* and the potential biotechnological applications for the bioconversion of lipid-rich by-products into highly desirable natural flavours. The objective of this work was to optimize a procedure for the extraction and partial purification of a LOX extract from *M. esculenta* and to characterize the LOX activity with respect to pH optima, substrate specificity, kinetic studies and end-product specificity.

2. Materials and methods

2.1. Culture growth and harvesting conditions

The culture of *M. esculenta* was grown according to a slight modification of the procedure described by Belinky et al. [32]. The culture medium consisted of 10 g of glucose, 0.068 g of KH_2PO_4, 8 ml of corn steep liquor, 2.5 g of soybean oil, and 100 ml of mineral solution in one liter of citrate buffer solution (0.01 M, pH 5.0); the mineral solution contained (in g l^{-1}): 2.3 g $MgSO_4.6H_2O$, 0.1 g $FeSO_4.7H_2O$, 0.023 g $MnSO_4.H_2O$, 0.06 g $ZnSO_4.7H_2O$, 0.08 g $CaCl_2.2H_2O$, and 0.03 g $CuSO_4.5H_2O$. One-week-old subculture containing the same culture medium was used as a source of inoculum. The subculture was homogenized three times for 10 s using an Ultrasonic Processor 2020 XL sonicator (Heat system, Farmingdale, NY). A portion of the resulting suspension was withdrawn and diluted with a 0.85 M NaCl solution until the absorbance of the diluted suspension was 0.500 at 600 nm; the culture medium was subsequently inoculated at 7% with the corresponding diluted suspension.

The culture was incubated at 25°C and 150 rev. min^{-1} for a period of 7 days. The biomass was harvested, washed with sodium phosphate buffer solution (0.01 M, pH 7.0), lyophilized and stored at -80°C.

2.2. Preparation of crude extracts

The lyophilized cells were ground to a fine powder using a mortar; the temperature was maintained at -40°C by the addition of liquid nitrogen during the grinding process. The mushroom powder was suspended in sodium phosphate buffer solution (0.01 M, pH 7.0) and subjected to glass beads homogenization for 3 min using a MSK cell homogenizer (Braun, Melsungen, Germany); the temperature of the homogenized suspension was maintained at 4°C by a gentle flow of CO_2 gas.

The homogenized extract was centrifuged (12 000 × *g*, 15 min) and the supernatant was lyophilized, whereas the pellet was discarded. The lyophilized enzymic extract was successively defatted with cold (-30°C) acetone and diethyl ether [40] and subjected to DNA precipitation, using protamine sulphate [41]. The subsequent fraction, considered to be the crude enzyme extract (FI), was subjected to further purification.

2.3. Partial purification of lipoxygenase

The partial purification of the LOX crude extract was initiated by the addition of solid ammonium sulphate at 0–60% saturation. The suspension was allowed to stand for 30 min and then centrifuged (12 000 × *g*, 15 min) to obtain the precipitate (FIIa). The supernatant was recovered and the same procedure was repeated twice to obtain the 60–80 and 80–100% ammonium sulphate saturation fractions (FIIb) and (FIIc), respectively. The precipitated enzyme fractions were resuspended in a minimum amount of sodium phosphate buffer solution (0.01 M, pH 7.0) and dialyzed against a diluted sodium phosphate buffer solution (0.001 M, pH 7.0) for a period of 12 h. The desalted enzymic suspensions were lyophilized, and subjected to kinetic studies.

2.4. Protein measurement

The protein concentration of the enzymic fractions was determined according to a modification of the Lowry method [42]. Bovine serum albumin (Sigma, St Louis, MO) was used as a standard for calibration.

2.5. Substrate preparation

Substrate standards used throughout this study, including 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]-glycerol), were purchased from Sigma. The preparation of stock solutions of substrates (4.0×10^{-3} M) was performed according to the procedure described previously [40].

2.6. Enzyme assay

LOX activity was measured spectrophotometrically according to the procedure outlined by Kermasha and Metche [40]. The reaction medium consisted of linoleic acid (50–600 μl) and a sufficient amount of selected

buffer solution (0.1 M) to adjust the final volume to 1 ml. The buffer, used for determination of enzyme activity in the crude extract and the partially purified extracts, was sodium phosphate buffer solution (0.1 M, pH 7.0). In addition, the investigation of the optimal pH for LOX activity was performed using the following buffer solutions: sodium citrate (0.1 M, pH 3.0), sodium acetate (0.1 M, pH 4.0–5.5), sodium phosphate (0.1 M, pH 6.0–8.0), Tris–HCl (0.1 M, pH 9.0), and sodium carbonate (0.1 M, pH 10.0). The enzymic reaction was initiated by the addition of the enzyme extract (25 μg protein). A control assay containing all the components, minus the enzyme preparation, was run in tandem with these trials. The reaction time was 3 min and all experiments were performed in triplicate. The LOX activity was measured on a Beckman DU-650 spectrophotometer (Beckman Instruments, San Raman, CA). The specific activity was expressed as the increase in A (mg protein)$^{-1}$ min^{-1} [50], where A is equal to 0.001 absorbance at 234 nm [43,44].

2.7. Recovery of hydroperoxides

The partially purified LOX extract (250 μg protein) of *M. esculenta* was incubated in 10.25 ml total reaction volume buffer mixture containing 1.5 ml linoleic acid (4 mM). After 12 min of incubation, the enzymic reaction was terminated by the addition of 4 M HCl to obtain a pH of 3.0. The HPODs were extracted with diethyl ether and all traces of protein and Tween-20 were removed by washing the extract with water. The diethyl ether was evaporated using a gentle stream of nitrogen. The polar oxygenated products including the HPODs were subsequently separated from the non-oxidized linoleic acid using a SPE silica column (Supelclean LC-Si 6 ml (0.5 g), Supelco, Bellefonte, PA) according to the procedure described by Toschi et al. [45].

2.8. High-performance liquid chromatography of the reduced hydroperoxides

The enzymically-catalyzed HPODs were dissolved in methanol and reduced to corresponding hydroxides of linoleic acid with $NaBH_4$ [10]. The high-performance liquid chromatography (HPLC) system used for the analyses of the reduced HPODs was Beckman Gold (Beckman Instruments), equipped with a UV diode-array detector (Beckman, Model 168) and fitted with a computerized data handling integrated system (Beckman model 126). UV detection was performed specifically at 234 nm. Injection was carried out with an automatic injector (Varian, Autosampler 9095, Varian Associates, Walnut Creek, CA) fitted with a 20-μl loop. Separation of the reduced HPODs was performed on a normal-phase Alphabond silica column (300 × 3.9 mm, 5 μm) (Altech Associates, Deerfield, IL) using the eluant system composed of a mixture of hexane/diethyl ether/acetic acid (1000:200:1, v/v/v) at a flow rate of 1.0 ml min^{-1}. The reduced HPOD isomers were subsequently collected separately and subjected to derivatization.

2.9. Derivatization of reduced hydroperoxides

The reduced HPODs were successively methylated with diazomethane, hydrogenated with platinum oxide and silylated with bis(trimethylsilyl)trifluoroacetamide (BSTFA) to produce the corresponding methyl trimethylsilyloxystearate (MTMS) derivatives as described by Bisakowski et al. [10].

2.10. Gas chromatography/mass spectrometry analyses of derivatized hydroperoxides

The GC/MS system used for the analyses of the treated HPODs was a HP 6890 Series GC System (Hewlett Packard, Avondale, PA) with computerized integration and data handling, and a 5973 Mass Selective Detector (Hewlett Packard). Injection was done through an automatic liquid sampler (HP 6890 Series Injector, Hewlett Packard); the volume analyzed was 1 μl by pulsed splitless mode injection using an inlet pressure of 25 psi for 1.50 min after each injection. Separation of the treated HPODs was done on a fused silica capillary HP-5MS column (30 m × 0.25 mm × 0.25 μm film thickness, Hewlett Packard); the initial column temperature was 150°C and increased at a rate of 3°C min^{-1} to 250°C, followed by a rate of 10°C min^{-1} to a maximum of 290°C where it was held for 3 min. Flow rates were set at 30, 400 and 10 ml min^{-1} for the hydrogen, air and make-up gas (He), respectively; however, the carrier gas (He) flow rate was kept at 25 psi. Injector and detector temperatures were set at 250 and 300°C, respectively.

3. Results and discussion

3.1. Partial purification of the enzymic extract

Table 1 shows the partial purification of the crude LOX extract (FI) from *M. esculenta*. The results (Table 1) indicate that the partially purified enzymic fraction (FIIa) obtained at 0–60% ammonium sulphate precipitation showed an 8- to 10-fold higher specific activity than that exhibited by fractions (FIIb) and (FIIc) obtained at 60–80 and 80–100% of ammonium sulphate saturation, respectively. Fraction (FIIa) also had the highest recovery of 12.8% compared to that of 1.5 and 0.3% for fractions (FIIb) and (FIIc), respectively. On the basis of these findings, fraction (FIIa) was selected as the partially purified enzymic extract for further characterization studies.

Table 1
Partial purification of lipoxygenase from *M. esculenta*

Fraction	Specific activity[a]	Total activity[b]	Recovery (%)	Purification (fold)
Crude extract (FI)	482	1 735 200	100.0	1.00
Precipitation at ammonium sulphate saturation of				
0–60% (FIIa)	1084	224 035	12.8	2.25
60–80% (FIIb)	158	25 518	1.5	0.33
80–100% (FIIc)	94	4891	0.3	0.20

[a] Specific activity is defined as A mg protein^{-1} min^{-1}, where A is equal to 0.001 absorbance at 234 nm.
[b] Total activity is defined as A min^{-1}, where A is equal to 0.001 absorbance at 234 nm.

Recent reports indicate that similar ammonium sulphate concentrations were used to obtain partially purified LOX extracts from microbial sources. A crude suspension from *G. candidum* [8], *F. proliferatum* [9] and the green alga *Chlorella prenoidosa* [46] was partially purified using ammonium sulphate precipitation at 10–40, 0–40 and 0–42%, respectively while that from *S. parasitica* [7], *Gracilariopsis lemaneiformis* [47] and *G. graminis* [18] was partially purified at 25–55, 30–55 and 20–45% of saturation, respectively.

3.2. Effect of pH on LOX activity

Fig. 1 shows the effect of pH on the LOX activity of the enzymic preparation from *M. esculenta*, determined over a wide pH range of 3.0–10.0. The highest LOX activity was obtained at the acidic pH of 3.0 and a gradual decrease in enzymic activity was observed with an increase in pH value; LOX activity decreased by 50% at pH 6.0, followed by a decrease of 87.5% at pH 7 and of 100% at pH 9.0. Although the poor water solubility of linoleic acid in the acidic reaction media was improved by the addition of emulsifiers such as Tween-20 [50–52], further characterization studies of the LOX activity of fraction (FIIa) were performed at the relatively more neutral pH of 6.0 which allowed a better solubilization of the free linoleic acid [53]. Similar findings were reported for a partially purified extract from *G. candidum* [8] and *C. pyrenoidosa* [39], where the highest LOX activity was exhibited at pH 3.75 and 4.5, respectively.

Optimal LOX activity in the neutral and alkaline pH range has also been reported in the literature. A partially purified extract from the green alga *C. pyrenoidosa* [46], *E. intestinalis* [48] and *U. lactuca* [49] demonstrated optimal activity at pH 7.4, 7.8 and 7.5, respectively. A purified LOX extract from *S. cerevisiae* [54] and *Thermoactinomyces vulgaris* [55] showed maximal activity at pH 6.3 and 6.0. Optimal LOX activities were obtained at pH 6.0 and 10.5 for the purified LOX fractions from *F. proliferatum* [11] and at pH 8.0 and 10.0 for the enzymic activity of both of the partially purified extracts of *F. oxysporum* [14] and *S. cerevisiae* [38].

3.3. Substrate specificity studies

The substrate specificity of the LOX activity of the partially purified fraction (FIIa) was investigated (Table 2) using a wide range of substrates, including free fatty acids and mono-, di- and triacylglycerols. The highest relative enzymic activity was obtained with linoleic acid (100%) as substrate, followed by arachidonic acid (83.3%). The results also show that approximately only one-third of the relative LOX activity was demonstrated towards linolenic acid as substrate. The lowest relative LOX activity of 7.7–17.8% was exhibited towards the mono-, di- and triacylglycerols.

Similar findings were obtained for the enzyme activity of the partially purified extracts of *F. oxysporum*

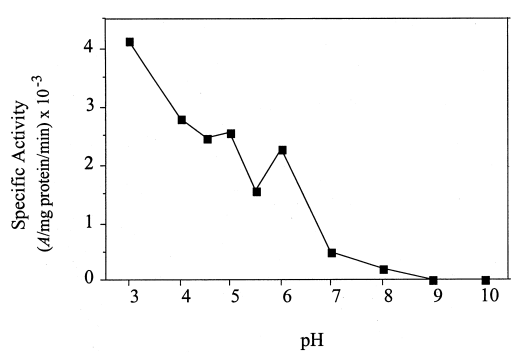

Fig. 1. Effect of pH on the lipoxygenase activity of the partially purified extract of *M. esculenta*. Specific activity was defined as A mg protein^{-1} min^{-1} where A was equal to 0.001 absorbance at 234 nm.

Table 2
Substrate specificity of lipoxygenase from *M. esculenta*

Substrate	Relative activity (%)[a]
Linoleic acid	100.0
Linolenic acid	29.2
Arachidonic acid	83.3
Monolinolein	10.1
Dilinolein	7.5
Trilinolein	17.8

[a] The relative percentage activity is defined as the lipoxygenase activity exhibited towards the substrate divided by that obtained with linoleic acid, multiplied by 100.

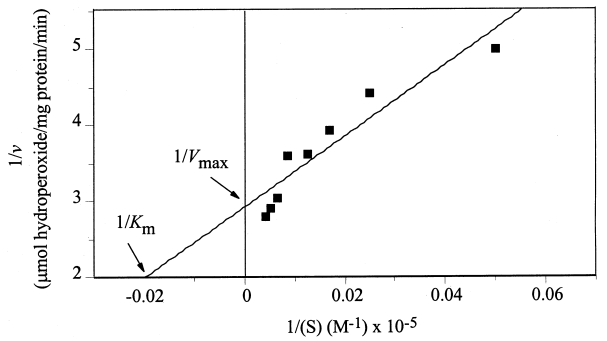

Fig. 2. Lineweaver–Burk plot of $1/v$ versus $1/(S)$ for the lipoxygenase activity of the partially purified extract of *M. esculenta*.

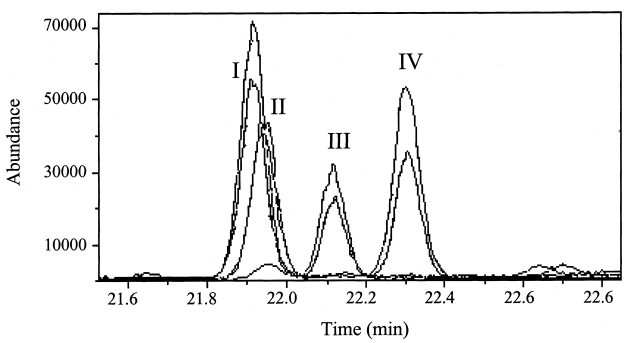

Fig. 3. Mass fragmentogram of the GC-MS analysis of the methyl trimethylsilyloxystearate (MTMS) derivatives of the hydroperoxides of linoleic acid, detected using their respective m/z ions of (I) 229 and 259 for 9-MTMS, (II) 215 and 273 for 10-MTMS, (III) 187 and 301 for 12-MTMS and (IV) 173 and 315 for 13-MTMS, produced by the enzymic extract of *M. esculenta*.

[14], *S. cerevisiae* [38] and *C. pyrenoidosa* [39], where the highest activity was exhibited using linoleic acid (100%) as substrate and only 15–30% with linolenic acid, followed by a low activity (0.3–10.6%) with mono-, di- and trilinolein. The purified LOX from *Th. vulgaris* [55] also demonstrated the highest substrate specificity with linoleic acid (100%), followed by linolenic acid (33%), but showed a lower activity towards arachidonic acid (22%). The LOX activity of the partially purified fraction from *F. proliferatum* [9] showed the highest affinity towards linoleic acid (100%) and relatively good activity (35.3–40.7%) towards linolenic acid and mono-, di- and trilinolein.

In contrast, Perraud et al. [8] reported that at pH 3.75, the LOX of the partially purified *G. candidum* fraction showed the highest activity towards linolenic acid (132%), followed by linoleic acid (100%) and relatively good (71–85%) activity towards arachidonic acid and mono-, di- and trilinolein whereas at pH 8.0, the enzyme activity showed the highest substrate preference towards linoleic acid and arachidonic acid (100–101%), followed by linolenic acid (42%) and little affinity (3–27%) towards mono-, di- and trilinolein. The LOX activity of the partially purified extract from *C. pyrenoidosa* [46] was 30% more active with linolenic acid than linoleic acid.

3.4. Kinetic studies

The V_{max} and K_m values (Fig. 2) for the LOX activity were calculated from the Lineweaver–Burk plots of $1/v$ versus $1/[S]$. The results indicate that a V_{max} value of 0.341 μmol hydroperoxide mg protein^{-1} min^{-1} and a K_m value of 1.59×10^{-4} M were obtained which corresponded to an overall catalytic efficiency of 2144. A purified mitochondrial fraction from *S. cerevisiae* [54] and a partially purified fraction from *G. candidum* [8], at pH 3.75 and 8.0, showed a similar K_m value of 2.86×10^{-4}, 0.67×10^{-4} and 1.059×10^{-4} M, respectively, for the enzymic activity. Slightly higher K_m values of 1.69×10^{-3} and 1.0×10^{-3} M and slightly lower K_m values of 9.12×10^{-5} and 5.15×10^{-5} M were reported for the enzyme activity from enzymic extracts of *F. oxysporum* [13], *Th. vulgaris* [55], *C. pyrenoidosa* [39] and *F. proliferatum* [9], respectively. The differences in the K_m values reported in the literature may be due to the different sources used to obtain the enzymes as well as variations in the procedures used to purify the enzyme extracts and the assay methods employed to detect and measure LOX activity [56].

3.5. Characterization of lipoxygenase end-products

Fig. 3 shows the mass fragmentogram of the GC-MS analysis of the MTMS derivatives of the HPODs enzymically-catalyzed by the LOX activity of the *M. esculenta* extract (FIIa). The results indicate that the MTMS derivatives of the HPODs eluted in numeric order beginning with the 9- and 10-HPODs, followed by the 12-HPOD and then by the 13-HPOD with respective retention times of 21.92, 21.95, 22.13 and 22.31 min; the HPOD isomers were identified via spectral match with HPOD standards and GC retention times [8,10]. Similar findings were reported previously by Bisakowski et al. [10]; the mass spectra of the 9-, 10-, 12-, and 13-MTMS derivatives of the HPOD isomers showed two intense ions of m/z at 229 and 259, 215 and 273, 187 and 301, and 173 and 315, respectively, characteristic of the respective fragmentation patterns.

Table 3 shows the relative qualitative production of HPODs by the LOX activity of the partially purified fraction (FIIa). Table 3 indicates that linoleic acid was converted predominantly into the corresponding 9-HPOD (36%) by the LOX activity of the partially purified extract. In addition, the production of approximately equal amounts of the 10- and 13-HPODs (24–26%) were also enzymically-catalyzed from linoleic acid followed by a low amount of the 12-HPOD (14%).

The LOX-catalyzed bioconversion of linoleic acid into similar HPOD isomers has been reported in the

Table 3
GC analyses of hydroperoxide isomers produced from linoleic acid by lipoxygenase from *M. esculenta*

	Relative peak area (%)[a] of hydroperoxide isomers
9-HPOD[b]	36
10-HPOD[b]	24
12-HPOD[b]	14
13-HPOD[b]	26

[a] The relative percentage peak area is defined as the peak area of the methyl trimethylsilyloxystearate isomer divided by the sum of the methyl trimethylsilyloxystearate isomers, multiplied by 100.
[b] HPOD, hydroperoxide isomer.

literature. Perraud et al. [8] reported that at pH 3.75 and 8.0, the LOX activity produced the 9- and 13-HPOD isomers at a ratio of 43.6:56.4 and 50:50, respectively. Bisakowski et al. [11] reported that the partially purified LOX fraction from *F. oxysporum* produced mainly the 13-HPOD at pH 6.0 and the 9- and 13-HPODs, at a ratio of approximately 1:1, at pH 10.5; the production of 10- and 12-HPODs was also suggested at pH 10.5. A LOX activity from *Oscilloria* sp. [57], *C. pyrenoidosa* [46], *E. intestinalis* [48], *U. lactuca* [49], and *S. cerevisiae* [54] catalyzed the bioconversion of linoleic acid into the 9- and 13-HPODs at a ratio of 48:52, 20:80, 66:34, 86:14 and 50:50, respectively. Grosch and Wurzenberger [58] reported the production of a 10-HPOD using linoleic acid by the LOX-type enzyme from the edible mushroom *P. bispora*.

In addition to the 9-, 10-, 12-, and 13-HPOD isomers, the production of other HPOD isomers of linoleic acid by LOX activity has been reported. A manganese LOX obtained from the filtrate of the culture medium of *G. graminis* converted linoleic acid into the 13(*R*)-HPOD (80%) and 11(*S*)-HPOD (20%) [21,59]. A dioxygenase activity in the purified and crude extracts of the fungi *G. graminis* [15–18] and *L. arvalis* [19], respectively, was reported to transform linoleic acid into 8*R*-HPOD.

4. Conclusion

The results obtained in this study showed that the LOX activity of the *M. esculenta* extract possessed similar characteristics to those found in other sources. In addition, the LOX activity showed a wide range of substrate and end-product specificity.

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