Characterization of Purified Lipoxygenase Extracts from *Fusarium proliferatum*

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A crude lipoxygenase (FI) extract from *Fusarium proliferatum* was partially purified by ammonium sulfate precipitation (FII). The purification of fraction FII by size-exclusion chromatography resulted in three major peaks, FIIIa, FIIIb, and FIIIc. Fraction FIIIa demonstrated the highest specific lipoxygenase activity as well as the highest recovery and was therefore further purified. The purification procedure resulted in one major fraction (FIV) by ion-exchange chromatography, and the presence of one major and two minor protein bands in both the native and sodium dodecyl sulfate−polyacrylamide gel electrophoresis patterns. The purified enzyme demonstrated approximately 2.5 times more activity toward mono- and dilinolein and 1.5 times more with linolenic and arachidonic acids than that exhibited toward linoleic acid at pH 6.0; however, at pH 10.5, this enzymatic fraction possessed an overall preference toward linoleic acid. The purified fraction produced mainly the 13-hydroperoxides (HPODs) from linoleic acid at pH 6.0 and the 9- and 13-HPODs, at a ratio of approximately 1:1, at pH 10.5. In addition, the presence of a lipoxygenase activity producing the 10- and 12-HPODs was also suggested at pH 10.5.

Keywords: *Lipoxygenase; Fusarium proliferatum; purification; characterization*

INTRODUCTION

Lipoxygenase (LOX; EC 1.13.11.12) is an enzyme that metabolizes linoleic acid and other polyunsaturated fatty acids containing a *cis*,*cis*-1,4-pentadiene moiety to hydroperoxy fatty acids by hydrogen abstraction from the methylene carbon and antarafacial insertion of molecular oxygen (Yamamoto, 1992). LOX has been reported in plant (Mack et al., 1987), animal (Yamamoto, 1983), and microbial (Mukherjee, 1951; Shimahara and Hashizume, 1973) sources. Theorell et al. (1947) were the first to isolate and purify the enzyme from soybeans; however, it was in the 1970s that Axelrod first separated and purified individual LOX isozymes from soybean seeds (Axelrod, 1974). In most sources, LOX exists as several isozymes that can differ significantly in properties such as optimum pH, substrate specificity, and end-products (O'Connor and O'Brien, 1991).

LOX-like activity was first described in several microorganisms, including *Aspergillus* and *Penicillium* (Mukherjee, 1951) and *Pseudomonas* and *Achromobacter* (Shimahara and Hashizume, 1973); however, apart from studies performed on plant and animal sources, little is known about LOX isozymes in microorganisms. Two LOX purified fractions were reported from *Saccharomyces cerevisiae* (Shechter and Grossman, 1983) and the thermophilic actinomycete, *Thermoactinomyces vulgaris* (Iny et al., 1993a). A LOX isozyme was also purified from *Fusarium oxysporum* (Matsuda et al., 1976). In addition, a purified LOX preparation was obtained from green algae *Oscillatoria* sp. (Beneytout et al., 1989) and the red algae *Gracilariopsis lemaneiformis* (Hamberg and Gerwick, 1993).

LOX plays an important role in the taste and flavor of food since the resultant end-products of the LOX-catalyzed reaction, that is, hydroperoxylinoleic acids (HPODs), can be converted to volatile compounds such as alcohols, ketones, and aldehydes that contribute to the organoleptic properties of food (Sessa, 1979).

The specific objective of this work was the development of a procedure for the purification and the characterization of LOX isozymes from *F. proliferatum* (Bisakowski et al., 1995) in terms of pH, kinetic parameters, substrate specificity toward linoleic, linolenic, and arachidonic acids as well as mono-, di-, and trilinolein, end-product specificity using linoleic acid as a model substrate, and the native and sodium dodecyl sulfate (SDS) electrophoretic profile.

MATERIALS AND METHODS

Culture Growth and Harvesting Conditions. The biomass culture of *F. proliferatum* was grown for 3 days on 2 L of modified Shoun medium, consisting of a mixture of 2 g of $NaNO_3$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 g of soya flour, 1 mL of mineral salt solution, 0.5 g of yeast extract, 1 g of $(NH_4)_2HPO_4$, and 125 mL of soya oil, at 27 °C and 85 rpm (Shoun et al., 1983). The fungal mycelia were harvested, washed with deionized water followed by a sodium phosphate buffer solution (0.01 M, pH 7.0), suspended in the phosphate buffer solution, and homogenized according to the procedure of Bisakowski et al. (1995). The enzyme extract was successively defatted with

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cold (−20 °C) acetone and diethyl ether (Kermasha and Metche, 1986) to remove the lipids, thereby eliminating their interference in the subsequent steps of purification. The defatted enzyme extract was suspended (1:10, w/v) in sodium phosphate buffer solution (0.01 M, pH 7.0) and subjected to mechanical stirring for 16 h. All purification procedures were performed at 4 °C unless otherwise indicated. The suspension was centrifuged (12000*g*, 15 min), and the supernatant was subjected to DNA precipitation with the use of protamine sulfate (Badaracco et al., 1983). The subsequent suspension was centrifuged (40000*g*, 10 min), and the supernatant, considered to be the crude enzyme extract (FI), was subjected to partial purification by the addition of solid ammonium sulfate at 40% of saturation (Bisakowski et al., 1995), thereby obtaining the partially purified LOX extract (FII).

Size-Exclusion Chromatography (SEC). The purification of the partially purified extract (FII) was carried out by SEC, using a Superose-12 HR 10/30 column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and the fast protein liquid chromatography (FPLC, Pharmacia) system. The column was equilibrated with sodium phosphate buffer solution (0.01 M, pH 7.0), and the sample (50 mg protein/mL) was solubilized in 200 μL of the buffer solution. Elution was performed at a flow rate of 0.5 mL/min, and 1 mL fractions were collected. The separated fractions were dialyzed against sodium phosphate buffer solution (0.001 M, pH 7.0) and lyophilized.

Ion-Exchange Chromatography (IEC). The main fraction obtained by SEC was subjected to IEC on the Mono Q HR 5/5 ion-exchange column (Pharmacia LKB Biotechnology) using the FPLC system. The column was pre-equilibrated with sodium phosphate buffer solution (0.005 M, pH 7.0; buffer A), and the sample (125 mg of protein/mL) was solubilized in 200 μL of the sodium phosphate buffer solution (0.005 M, pH 7.0). A linear gradient of buffer A and buffer B (buffer A containing 1 M NaCl) was used for elution at a flow rate of 1 mL/min. The separated fractions of 1 mL were dialyzed against sodium phosphate buffer solution (0.001 M, pH 7.0) and lyophilized.

Protein Determination. The protein concentration of the enzymatic fractions was determined according to the Lowry method as described by Hartree (1972). Bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used as a standard for calibration.

Substrate Preparation. The substrate selectivity of the purified LOX fractions was determined using standards, purchased from Sigma, which included linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid), linolenic acid (*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid), arachidonic acid (5,8,11,14-eicosatetraenoic acid), monolinolein [1-mono[(*cis*,*cis*)-9,12-octadecadienoyl]-*rac*-glycerol], dilinolein [1,3-di[(*cis*,*cis*)-9,12-octadecenoyl]-*rac*-glycerol], and trilinolein [1,2,3-tri[(*cis*,*cis*,*cis*)-9,12,15-octadecadienoyl]-*rac*-glycerol]. Stock solutions were prepared at a concentration of 4×10^{-3} M, as described previously (Kermasha and Metche, 1986).

Enzyme Assay. The LOX activity of the purified extracts of *F. proliferatum* was assayed spectrophotometrically (Beckman DU-650, Beckman Instruments Inc., Irvine, CA) according to the procedure described by Bisakowski et al. (1995) using linoleic acid [$(0.2-6) \times 10^{-6}$ M] as substrate. The enzyme assays were performed at the optimum pH for each of the fractions investigated; all of the fractions showed optimal LOX activity at pH 6.0, and fractions FIIIa, FIIIb, FIIIc, and FIV also exhibited maximal activity at pH 10.0, 7.5, 10.0, and 10.5, respectively. A control solution, containing all of the components without the enzyme preparation, was run in tandem with these trials. The formation of 9- and 13-HPODs by LOX activity was measured by an increase in absorbance at 234 nm (Surrey, 1964) due to the presence of a conjugated hydroperoxydiene moiety; however, the absence of this moiety in the 10- and 12-HPODs allowed the detection of these two products only as their respective methyl trimethylsilyloxystearate (MTMS) derivatives using the gas chromatography/mass spectroscopy (GC/MS) analyses. The specific activity of LOX was defined as the increase in U (mg of protein)$^{-1}$ min^{-1}, where U is equal to 0.001 absorbance at 234 nm (Ali Asbi et al., 1989).

Polyacrylamide Gel Electrophoresis (PAGE). Native PAGE was performed using a horizontal PhastSystem apparatus (Pharmacia LKB Biotechnology). The electrophoretic run and staining of the separated protein bands were performed as described previously by Bisakowski et al. (1995). SDS−PAGE was performed in a similar manner as described above for the native PAGE; however, the standards and samples were treated with SDS (2.5%) and 2-mercaptoethanol (5%) at 100 °C (10 min), and PhastGel SDS buffer strips (Pharmacia LKB Biotechnology), consisting of 0.20 M Tricine, 0.20 M Tris (pH 8.1), and 0.55% SDS in 3% agarose isoelectric focusing buffer, were used.

Preparation of Hydroperoxide Standards. The HPOD standards, the 9- and 13-HPODs, were prepared according to the procedure described by Kermasha et al. (1986). The HPODs were obtained by the incubation of commercial linoleic acid and soybean LOX-1 (Sigma). The reaction medium consisted of 22.4 mg of linoleic acid in which the final concentration of the substrate in the reaction medium was equal to 2 mM, 0.6% (v/v) polyoxyethylene sorbitan monolaurate (Tween-20), and sufficient Tris-HCl buffer solution (pH 7.3, 0.1 M) to adjust the final volume of the mixture to 40 mL. The reaction medium was held at 25 °C in a temperature-controlled water bath with agitation at 100 rpm, and the HPODs were subsequently extracted with diethyl ether.

Recovery of Hydroperoxides. To recover adequate quantities of HPODs by the activity of the purified enzymatic extracts, a scale-up of the enzymatic assay from 100 μL to 3 mL was performed. A control solution, containing all of the components minus the enzyme preparation, was run in tandem with these trials.

HPLC of End-Products. The high-performance liquid chromatography (HPLC) system used for the analyses of HPODs was a Beckman Gold (Beckman Instruments) with computerized integration and data handling (Beckman model 126), equipped with a Beckman diode array UV detector (model 168). Injection was achieved through an automatic injector (Varian, model 9095, Walnut Creek, CA) fitted with a 20 μL loop. The HPODs were separated on a reversed-phase Econosil C_{18} column (250 mm × 4.6 mm i.d., Alltech Associates Inc., Deerfield, IL) and monitored by their specific absorption at 234 nm; the eluant system was a mixture of methanol/water/acetic acid (75:24.95:0.05, v/v/v) at a flow rate of 1 mL/min.

Derivitization of Linoleic Acid Hydroperoxides. The HPODs were resolubilized in 100 μL of methanol and reduced to hydroxylinoleic acids (HODEs) by the addition of sodium borohydride ($NaBH_4$) solution according to the procedure described by Bisakowski et al. (1997).

The carboxyl group of HODEs was methylated, using the Aldrich MNNG-diazomethane kit (Aldrich Chemical Co., Milwaukee, WI), and the double bonds were subsequently hydrogenated using platinum dioxide (15 mg) and a gentle flow of hydrogen gas (Bisakowski et al., 1997).

Finally, silylation of hydroxyl groups to trimethylsilanes was performed by resolubilizing the residual extract in pyridine (50 μL) and *N*,*N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (100 μL) and allowing the reaction to occur for 30 min at 40 °C (Kermasha et al., 1986).

Gas−Liquid Chromatography/Mass Spectometry (GC/MS) of End-Products. The GC/MS analyses of the treated HPODs were performed with an HP 6890 Series GC system (Hewlett-Packard Co., Palo Alto, CA) equipped with a mass selective detector (Hewlett-Packard). A fused silica capillary SPB-1 column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco Inc., Bellefonte, PA) was used as a stationary phase using the conditions described by Bisakowski et al. (1997). Mass spectra were obtained by electron impact (EI) ionization at 70 eV.

Capillary Electrophoresis (CE) Analyses. The CE system used was the P/ACE System 5510 from Beckman Instruments Inc.. Electrophoresis separations were achieved using a neutral capillary of 37 × 50 μm i.d., an applied voltage

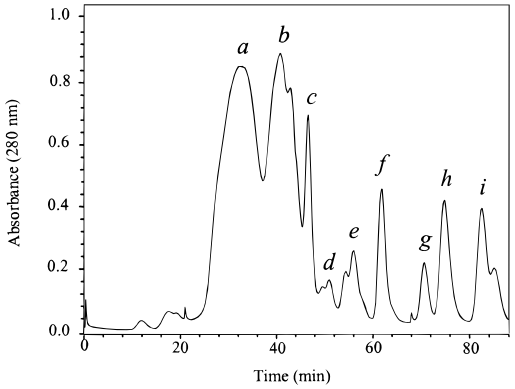

Figure 1. SEC of the partially purified LOX extract (FII) from *F. proliferatum* on the Superose-12 HR 10/30 column.

Table 1. Purification Scheme of LOX from *F. proliferatum*

fraction	total protein[a] (mg)	spec act.[b]	total act.[c]	recov (%)	purifn (-fold)
crude (FI)	112706	506	57029236	100.00	1
ammonium sulfate precipitation 0−40% (FII)	3026	3538	10705988	18.86	7
SEC					
FIIIa	77	5057	389389	0.69	10
FIIIb	23	3511	80753	0.14	7
FIIIc	13	1210	15730	0.03	2
IEC					
FIV	5	6636	33180	0.06	13

[a] Protein was determined according to a modification of the Lowry method (Hartree, 1972), using bovine serum albumin as a standard. [b] Specific activity is defined as A (mg of protein)$^{-1}$ min^{-1}, where A is equal to 0.001 absorbance at 234 nm. [c] Unit of activity is defined as A min^{-1}, where A is equal to 0.001 absorbance at 234 nm.

of 20.1 kV, and UV detection at 230 nm, at a temperature setting of 15 °C. The running buffer was a high pH buffer solution (pH 8.0) containing 15 mM β-cyclodextrin as outlined in the manual (Beckman Instruments Inc., 1994). Samples were injected for 5 s, using a pressure of 20 psi, and each analysis lasted 30 min. The capillary was washed, before each analysis, with 0.1 M HCl for 30 s, followed by a 2 min wash with water and a 2 min rinse with buffer.

RESULTS AND DISCUSSION

Enzyme Purification. The partially purified LOX extract was separated by SEC into nine fractions, FIIIa−FIIIi (Figure 1). The first three enzymatic extracts, FIIIa, FIIIb, and FIIIc, exhibited the highest LOX activity, possessing 88% of the total activity (data not shown). Fraction FIIIa exhibited 80% of the total activity present in fraction FIII and a 10-fold increase in purification with respect to the crude fraction FI (Table 1). The results also show that FIIIa possessed 1.4 and 4 times higher specific activity than that for fractions FIIIb and FIIIc, respectively, as well as 4.9 and 23 times the respective recoveries. On the basis of these findings, fraction FIIIa was selected for further purification using IEC.

Figure 2 indicates that the IEC of fraction FIIIa resulted in one main fraction (FIV), which eluted at 11 min. In addition, fraction FIV had a specific activity of 6636 U (mg of protein)$^{-1}$ min^{-1}, which was 13 times higher than that shown by the crude extract, that is, a 13-fold in purification (Table 1).

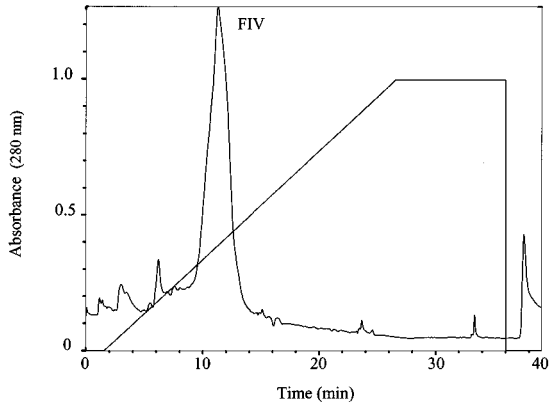

Figure 2. Profile of purification of fraction FIIIa from *F. proliferatum*, obtained from SEC, on the Mono Q HR 5/5 column, using the FPLC system.

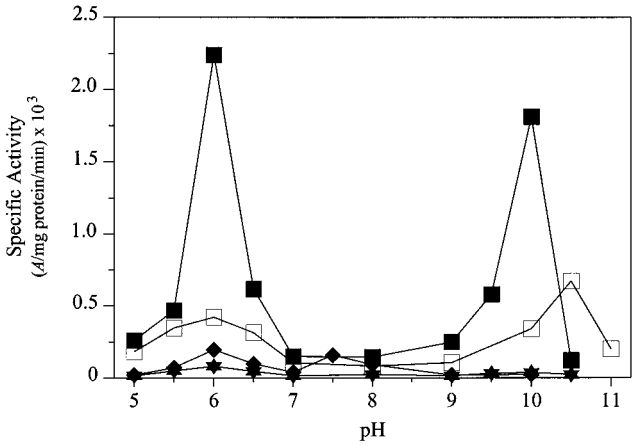

Figure 3. Effect of pH on the LOX activity of the purified LOX fractions obtained from *F. proliferatum*: FIIIa (■); FIIIb (◆); FIIIc (✱); FIV (□).

Matsuda et al. (1976) reported a LOX isozyme from *F. oxysporum* that exhibited a 465-fold increase in purification and 21% recovery. A LOX preparation from the green algae *Oscillatoria* sp. (Beneytout et al., 1989) was purified 81-fold and had a recovery of 0.089%. Two active LOX fractions from *Thermoactinomyces vulgaris* (Iny et al., 1993b) were separated using acetate (pH 5.0) and borate (pH 9.0) buffers; the former had a recovery and purification factor of 10.1% and 9.2-fold, respectively, while those of the latter were 3 times higher. In addition, the acetate fraction (pH 5.0) from *Saccharomyces cerevisiae* (Shechter and Grossman, 1983) exhibited an overall purification of 6.5-fold and a recovery of 26.9%, whereas the phosphate fraction (pH 6.85) was purified 9.7-fold and showed a recovery of 44.9%. Shimahara and Hashizume (1973) reported a 25-fold purified LOX-like enzymatic fraction from *Pseudomonas aeruginosa*. The discrepancy regarding the various degrees of purification of LOX isozymes could be due to the different methods used for the extraction and purification of these enzymes from various sources (Whitaker, 1972).

Optimum pH. Figure 3 demonstrates the influence of pH on the LOX activity of purified fractions. Fractions FIIIa, FIIIb, FIIIc, and FIV demonstrated an optimal activity at the more neutral pH of 6.0; the partially purified fraction FII (Bisakowski et al., 1995) also exhibited maximal LOX activity at this pH. In addition, the presence of optimal LOX activity was

Table 2. Substrate Specificity of the Purified LOX Fractions from *F. proliferatum*

		rel LOX activity[a] on substrate					
fraction	pH	linoleic acid	linolenic acid	arachidonic acid	monolinolein	dilinolein	trilinolein
FIIIa	6.0	100	44	38	12	28	46
	10.0	100	52	11	3	9	1
FIIIb	6.0	100	65	92	140	122	115
	7.5	100	54	58	91	44	112
FIIIc	6.0	100	30	21	13	6	8
	10.0	100	74	10	21	6	19
FIV	6.0	100	167	154	250	222	67
	10.5	100	27	12	68	80	71

[a] Relative activity is defined as the percentage of specific activity, defined as A (mg of protein)$^{-1}$ min^{-1}, where A is equal to 0.001 absorbance at 234 nm, compared to that obtained with linoleic acid.

exhibited at pH 10.0 for fractions FIIIa and FIIIc, at pH 10.5 for fraction FIV, and at pH 7.5 for fraction FIIIb. The enzymatic extract of fraction FIIIa possessed the highest specific activity at its two pH optima, followed by that of the more purified fraction FIV; this relative loss of enzymatic activity during the course of purification is not straightforward due to the fragile nature of LOXs. The reason for enzyme activity loss is unknown; however, proteolysis and loss of catalytic iron after exposure to oxygen in the absence of reducing agents have been implicated (Percival, 1991). Some LOXs are therefore purified under anaerobic conditions (Carroll et al., 1993).

A purified LOX extract from *T. vulgaris* (Iny et al., 1993b) exhibited maximal activity at pH 6.0 and a relatively lower one at pH 11.0, which could be indicative of the presence of two isozymes. A purified LOX extract from *S. cerevisiae* (Shechter and Grossman, 1983) and *Oscillatoria* sp. (Beneytout et al., 1989) exhibited maximal activity at pH 6.3 and 8.8, respectively. A pH of 10.8−11.0 was reported for the isolated LOX from *P. aeruginosa* (Shimahara and Hashizume, 1973) and the *Bacillus* species (Shimahara, 1964), whereas the purified isozyme from *F. oxysporum* exhibited an optimum of activity at pH 12.0, with a shoulder peak at pH 10.0 (Matsuda et al., 1976).

Enzyme Specificity. Table 2 shows that fractions FIIIa and FIIIc exhibited a strong substrate specificity toward linoleic acid at pH 6.0 and 10.0 in comparison with the other substrates. In contrast, fraction FIIIb showed an increased preference toward monolinolein (140%), dilinolein (122%), and trilinolein (115%) at pH 6.0 and trilinolein (112%) at pH 7.5 in comparison to linoleic acid (100%); fraction FIIIb was also more active toward linoleic acid in comparison to linolenic and arachidonic acids, at both pH optima. The purified ion-exchange LOX fraction (FIV) demonstrated an overall increased activity, at pH 6.0, toward linolenic (167%) and arachidonic (154%) acids as well as monolinolein (250%) and dilinolein (222%) but was considerably less active toward trilinolein (67%); however, at pH 10.5, fraction FIV showed a strong preference for linoleic acid (100%) over linolenic (27%) and arachidonic (12%) acids but exhibited similar activity toward monolinolein (68%), dilinolein (80%), and trilinolein (71%). The partially purified extract FII of *F. proliferatum* (Bisakowski et al., 1995) exhibited maximal activity toward linoleic acid and ≈37% of that activity toward mono-, di-, and trilinolein.

The purified LOX extract from *S. cerevisiae* (Shechter and Grossman, 1983) demonstrated similar activity toward linoleic and arachidonic acids as substrates but only 40% of that activity toward linolenic acid. The purified enzyme extract from *T. vulgaris* (Iny et al., 1993b) showed substrate specificity toward linoleic acid (100%) but little activity toward linolenic (33%) and arachidonic (22%) acids. The LOX isozyme from *F. oxysporum* possessed 13.3 times higher activity toward linoleic acid in comparison to linolenic acid (Matsuda et al., 1976).

Table 3. Kinetic Parameters, Obtained by the Lineweaver−Burk Plot, for the Purified LOX Fractions of *F. proliferatum*

fraction	pH	K_m (10^{-6} M)	V_{max}[a]
SEC			
FIIIa	6.0	2.5	13.9
	10.0	4.7	20.6
FIIIb	6.0	4.2	1.0
	7.5	7.5	2.7
FIIIc	6.0	3.1	4.0
	10.0	4.7	3.4
IEC			
FIV	6.0	11.0	8.4
	10.5	3.9	6.6

[a] The V_{max} value is expressed as μmol of hydroperoxide (mg of protein)$^{-1}$ min^{-1}.

Kinetic Studies. Table 3 shows the V_{max} and K_m values obtained for the purified LOX preparations using the best straight line determined by the Lineweaver−Burk plots (Lineweaver and Burk, 1934). Fraction FIIIa exhibited the highest V_{max} values of 13.9 and 20.6 μmol (mg of protein)$^{-1}$ min^{-1} at pH optima of 6.0 and 10.0, respectively, followed by fraction FIV, which showed V_{max} values of 8.4 and 6.6 μmol (mg of protein)$^{-1}$ min^{-1} at pH optima of 6.0 and 10.0, respectively. The lowest values of 1.0 and 2.7 μmol (mg of protein)$^{-1}$ min^{-1} were displayed by the activity of fraction FIIIb, at pH 6.0 and 7.5, respectively. All purified factions except FIIIb (pH 6.0) exhibited higher V_{max} values in comparison to the V_{max} value of 1.6 μmol (mg of protein)$^{-1}$ min^{-1} of the partially purified extract. In addition, the purified fractions FIIIa, FIIIb, FIIIc, and FIV all showed a considerably higher affinity toward linoleic acid as substrate as indicated by their lower K_m values in comparison to the partially purified extract, which had a K_m value of 5.15×10^{-5} M.

Higher K_m values of 1.69×10^{-3} and 1.0×10^{-3} M were obtained for the LOX isozyme from *F. oxysporum* (Matsuda et al., 1976) and an enriched LOX preparation from *T. vulgaris* (Iny et al., 1993b), respectively. Shechter and Grossman (1983) reported a K_m value of 2.86×10^{-4} M for the purified LOX extract from *S. cerevisiae*.

Electrophoresis. Figure 4A shows the native PAGE electrophoretic pattern of the enzymatic fractions. The purification of the *F. proliferatum* extract FII by SEC resulted in the isolation of fraction FIIIa, which comprises seven protein bands. In addition, IEC of fraction

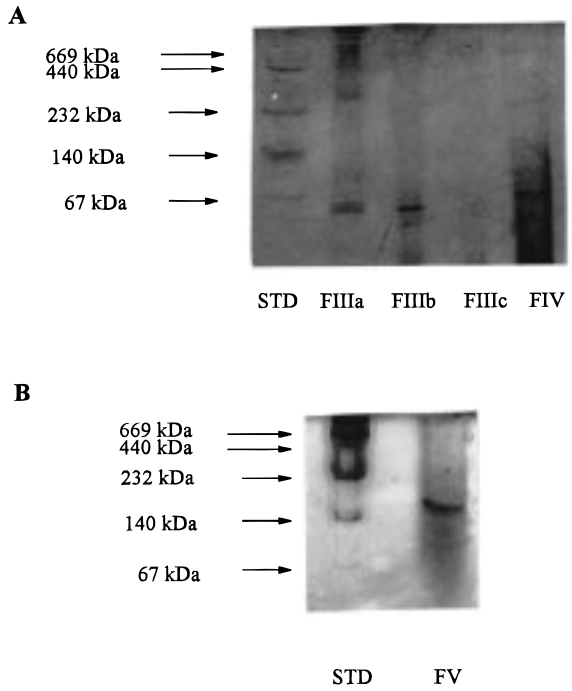

Figure 4. Native PAGE (A) and SDS−PAGE (B) of the purified LOX fractions obtained from *F. proliferatum* using the FPLC sytem.

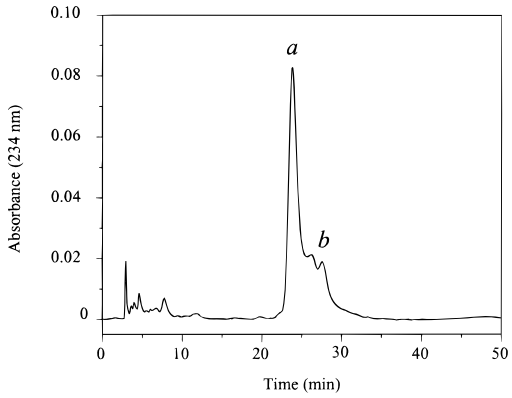

Figure 5. HPLC elution profile of the hydroperoxides of linoleic acid, produced by the purified enzymatic extract (FIIIa) obtained from *F. proliferatum* by SEC.

FIIIa resulted in the isolation of one major LOX protein fraction, FIV, with a relative molecular mass of 172 kDa; the efficiency of the purification of FIV is indicated by the presence of one major and two minor faint protein bands in both native PAGE and SDS−PAGE (Figure 4B).

The two purified LOX fractions from *S. cerevisiae* (Shechter and Grossman, 1983) showed three bands on polyacrylamide gel, a major band and two weak bands. In addition, both purified LOX preparations from *T. vulgaris* (Iny et al., 1993b) demonstrated the presence of one major protein with traces of another protein on PAGE. Matsuda et al. (1976) reported that the purified isozyme from *F. oxysporum* exhibited only one band on polyacrylamide gel in the presence and absence of SDS.

Enzymatic End-Products. Figure 5 shows the typical HPLC elution profile of the HPODs produced by the enzymatic fraction FIIIa; similar HPOD elution profiles were obtained for the purified fractions FIIIb and FIV as well as the partially purified extract (FII) of *F. proliferatum* (Bisakowski et al., 1997). The HPODs

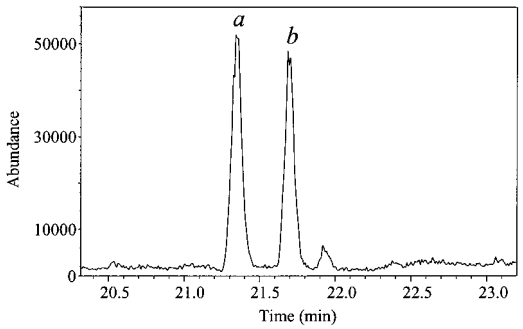

Figure 6. GC chromatograms of linoleic acid hydroperoxides (a) 9- and 10-MTMS and (b) 13-MTMS, produced by the purified enzymatic extract (FIIIa) obtained from *F. proliferatum* by SEC.

Table 4. Gas−Liquid Chromatography Analyses of Hydroperoxide Isomers, Produced by the Purified Extracts of *F. proliferatum*

		rel peak area[a] (%)	
fraction	pH	HPOD isomer 9	HPOD isomer 13
SEC			
FIIIa	6.0	—[b]	100.0
FIIIa	10.0	46.2	53.8
FIIIb	6.0	39.1	60.9
FIIIb	7.5	—[b]	100.0
IEC			
FIV	6.0	8.1	91.9
FIV	10.5	47.4	52.6

[a] The relative percentage peak area was defined as the peak area of the methyl trimethylsilyloxystearate isomer divided by the sum of the methyl trimethylsilyloxystearate isomers, multiplied by 100. [b] No peak detected.

eluted as one major peak (*a*) and one minor peak (*b*) consisting of two shoulders; the former was selected for further study as it was considered to be the major HPOD fraction.

The GC profile of the MTMS derivatives of HPODs obtained by the LOX activity of the purified fraction FIIIa is shown in Figure 6; the purified fractions FIIIb and FIV as well as the partially purified extract (FII) of *F. proliferatum* (Bisakowski et al., 1997) exhibited a similar elution profile. The results demonstrate that the two MTMS derivatives of the HPOD isomers were eluted by numeric order starting with the 9-HPOD, closely followed by the 13-HPOD at 21.3 and 21.7 min, respectively.

The mass spectra for the 9- and 13-MTMS derivatives of HPODs, produced using the purified fractions FIIIa, FIIIb, and FIV of *F. proliferatum*, were similar to those obtained by the partially purified extract (FII) (Bisakowski et al., 1997). The presence of the 9- and 13-MTMS derivatives of the HPODs was indicated by the characteristic fragmentation patterns resulting from α-cleavage of both sides of the carbon atom to which the trimethylsiloxy group is attached; the results show the presence of the 9-MTMS as indicated by the *m/e* fragments at 230 and 260 and that of the 13-MTMS as exhibited by the strong signals at 174 and 316 (Beneytout et al., 1989; Matsuda et al., 1978). The results (not shown) indicate that the 9- and 13-MTMSs produced from the 9- and 13-HPOD standards and by the LOX activity of the purified LOX fractions FIIIa, FIIIb, and FIV possessed the same characteristic mass spectra.

Table 4 shows the relative qualitative production of HPODs by the purified LOX fractions. The results show that fractions FIIIa, FIIIb, and FIV produced the 9- and

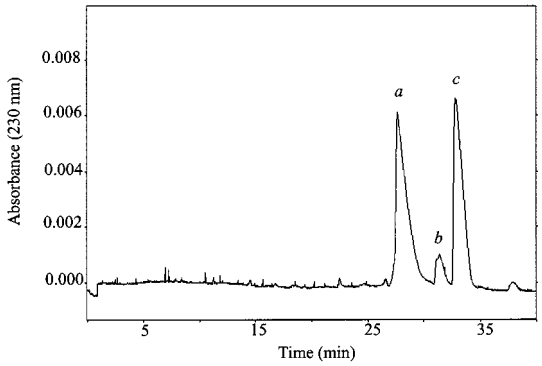

Figure 7. CE elution profile of hydroperoxides (HPODs) of linoleic acid, (a) 9-HPOD, (b) 10-HPOD, and 12-HPOD and (c) 13-HPOD, produced by the enzymatic extract (FIIIa) obtained from *F. proliferatum* by SEC.

13-HPODs at a ratio of 45.3−54.7% at pH 10.0, 6.0, and 10.5, respectively. However, the results also indicate a LOX activity in fractions FIIIa, FIIIb, and FIV, which produced the 13-HPOD as the predominant isomer at pH 6.0, 7.5, and 6.0, respectively. The production of the different ratios of HPOD isomers at two different pH optima suggests the presence of at least two LOX isozymes or at least one enzyme with two specificities in each purified fraction.

Matsuda et al. (1976) reported the conversion of linoleic acid into 9- and 13-HPODs by a LOX preparation from *F. oxysporum* (70:30 at pH 9.0 and 56:44 at pH 12.0). Iny et al. (1993a) showed that the LOX extract from the thermophilic actinomycete *T. vulgaris* produced the 9- and 13-HPODs at a ratio of 56:44. A LOX activity in the yeast *Saccharomyces vini* (Lyudnikova et al., 1984) and the mitochondrial fraction of *S. cerevisiae* (Shechter and Grossman, 1983) was reported to catalyze the bioconversion of linoleic acid into the 9- and 13-HPODs. In addition, a LOX extract converted linoleic acid into the 9- and 13-HPODs (48:52) for *Oscillatoria* sp. (Beneytout et al., 1989) and (20:80) for *Chlorella pyrenoidosa* (Zimmerman and Vick, 1974).

Figure 7 shows the CE elution profile of the HPODs produced by the purified fractions FIIIa, FIIIb, and FIV as well as the standards 9- and 13-HPODs. The results show that the HPODs were eluted in numeric order beginning with the 9-HPOD and ending with the 13-HPOD with respective retention times of 27.7 and 33 min. The results also indicate the presence of a 10- and 12-HPOD, as suggested by the minor peak between the 9- and 13-HPODs, possessing a retention time of 31.5; the presence of this minor peak remained undetected by GC as its concentration was too low.

Table 5 shows the relative qualitative production of HPODs by the purified LOX fractions as detected using CE. The results demonstrate that the relative proportions of HPOD isomers detected using CE are similar to those obtained using GC. However, due to the increased sensitivity of CE, fractions FIIIa (pH 6.0) and FIIIb (pH 7.5) also show the presence of the 9-HPOD isomer at relative peak areas of 34.9 and 27.9%, respectively. In addition, purified fractions FIIIa (pH 6.0), FIIIa (pH 10.0), FIIIb (pH 6.0), and FIV (pH 10.5) also suggest the presence of a LOX activity that produced the 10- and 12-HPOD at relative peak areas of 12.8, 5.1, 16.3, and 10.2%, respectively. However, fractions FIIIb (pH 7.5) and FIV (pH 6.0) did not exhibit the presence of such a LOX activity.

A LOX activity was also reported in several mushroom species, which specifically catalyzed the conversion of linoleic acid into a 10-HPOD (Wurzenburger and Grosch, 1984), whereas an enzyme preparation of the red alga *Lithothamnion corallioides* produced the 11-HODE and smaller amounts of 9- and 13-HODE as well as the 11-ketodienone of linoleic acid (Hamberg et al., 1992).

Table 5. CE Chromatography Analyses of Hydroperoxide Isomers, Produced by the Purified Extracts of *F. proliferatum*

		rel peak area[a] (%)		
fraction	pH[b]	HPOD isomer 9	HPOD isomer 10/12	HPOD isomer 13
SEC				
FIIIa	6.0	34.9	12.8	52.3
FIIIa	10.0	45.4	5.1	49.4
FIIIb	6.0	35.0	16.3	48.7
FIIIb	7.5	27.9	—[b]	72.1
IEC				
FIV	6.0	—[b]	—[b]	100.0
FIV	10.5	39.6	10.2	50.1

[a] The relative percentage peak area was defined as the peak area of the hydroperoxide divided by the sum of the hydroperoxide isomers, multiplied by 100. [b] No peak detected.

Conclusion. The results indicated that the purification of the partially purified extract of *F. proliferatum* resulted in the separation of several purified LOX activities, which differed in terms of their pH optimum, substrate specificity, and end-product specificity. Several of these purified LOX fractions exhibited characteristics, in terms of HPOD production and a strong preference toward linoleic acid as substrate, similar to those of LOX extracts from other sources. However, the results also show that a unique LOX activity was isolated as suggested by the biocatalysis of various HPOD isomers and its strong substrate specificity toward mono-, di-, and trilinolein.

ABBREVIATIONS USED

LOX, lipoxygenase; HPOD, hydroperoxylinoleic acid; SDS, sodium dodecyl sulfate; FPLC, fast protein liquid chromatography; IEC, ion-exchange chromatography; MTMS, methyl trimethylsilyloxystearate; GC/MS, gas−liquid chromatography/mass spectometry; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; HODE, hydroxylinoleic acid; CE, capillary electrophoresis.

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