Characterization of Lipoxygenase Extracts from *Penicillium* sp.

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ABSTRACT: Biomasses of *Penicillium camemberti* and *P. roqueforti* strains were grown and harvested after 10 d of incubation, a period that corresponded to the maximal dry weight of mycelium as well as to lipoxygenase (LOX) activity. Partially purified LOX extracts were obtained by ammonium sulfate precipitation of the crude enzymatic extracts that had been recovered from the biomasses. The partially purified LOX extracts exhibited a preferential specificity toward free fatty acids, including linoleic, linolenic, and arachidonic acids, rather than fatty acid acylglycerols, including mono-, di-, and trilinolein. The K_m and V_{max} values of LOX activity were investigated. Normal-phase high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry analyses showed that the LOX activity of the microbial extracts converted linoleic acid mainly into the corresponding 9- and 13-hydroperoxides (HPOD). However, the production of a significant proportion of 10-HPOD, ranging from 4 to 9% of the total HPOD, was also demonstrated. In addition, the enantiomeric ratios of the 9- and 13-HPOD produced were determined at both pH optima by chiral-phase HPLC. The results indicated that an almost racemic mixture had been obtained which can be related to either low enantioselectivity of LOX or the presence of isozymes showing complementary enantioselectivity.

Paper no. J9348 in *JAOCS 77,* 335–342 (April 2000).

KEY WORDS: Characterization, end products, lipoxygenase, *Penicillium* sp.

Lipoxygenase (LOX; EC 1.13.11.12) is a dioxygenase that catalyzes the oxygenation of polyunsaturated fatty acids containing a *cis*,*cis*-1,4-pentadiene moiety to hydroperoxides (HPOD) (1). Although the LOX of plants and animals have been extensively studied (2,3), less information is available regarding the characterization and the role of LOX in microorganisms. Since Mukherjee (4) first described a LOX-like activity in *Penicillium* and *Aspergillus*, this activity has been reported in other fungi, including *Fusarium* sp. (5,6), *Saprolegnia* sp. (7,8), *Pityrosporum orbiculare* (9), *Lagenidium giganteum* (10), *Achlya* sp. (11,12), *Gäumannomyces vulgaris* (13), *Geotrichum candidum* (14), and in the yeast *Saccharomyces* sp. (15,16). LOX also have been found in a few bacteria (17,18) as well as in algae (19–22). In addition, the enzyme was associated with the formation of volatile C_8 flavor compounds from linoleic and linolenic acids in edible mushrooms such as *Agaricus* sp. (23–25), *Pleurotus pulmonarius* (26,27), and in the fungus *Penicillium* sp. (28,29).

LOX play a key role in the generation of flavor compounds, such as carbonyl compounds and alcohols (30), by producing different HPOD isomers that are considered to be flavor precursors. Microbial LOX are of particular interest for the production of aroma compounds, since they display many unique specificities, producing a wide range of HPOD isomers (31).

The present work is part of ongoing research in our laboratory (6,14,31–35) aimed at the biotechnological applications of microbial enzymes in the biogeneration of natural flavors. The specific objective of this study was to investigate the presence of LOX in the fungi *Penicillium camemberti* and *P. roqueforti* as well as their partial purification and characterization with respect to their pH optima, substrate specificity, kinetic studies, and end product positional and stereospecificity.

MATERIALS AND METHODS

Culture growth and harvesting conditions. Penicillium sp. were provided by Dr. E. Spinnler (INA-GRIGNON, Paris, France). A medium, containing glucose (10.0 g/L), $NaNO_3$ (3.0 g/L), KH_2PO_4 (1.0 g/L), KCl (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), and $FeSO_4 \cdot 7H_2O$ (10.0 mg/L) was used for the culture of *P. camemberti*. The culture medium was adjusted to pH 6.0 with 1 M NaOH before sterilization at 120°C for 15 min. A similar medium also was prepared for the culture of *P. roqueforti*, except that KH_2PO_4 was replaced by K_2HPO_4 (1.0 g/L) and the initial pH was adjusted to 4.0 with 1 M HCl.

Spores of *P. camemberti* and *P. roqueforti* were produced on potato dextrose agar in Roux flasks. To prepare the inoculum, the spores were collected after 5 wk of incubation at 20°C by shaking the flasks with 50 mL of a sterilized solution of 5% polyoxyethylenesorbitan monooleate (Tween 80). The spores, suspended in the medium, were counted using a Neubauer Counting Chamber (Hausser Scientific, Harsham, PA).

The strains were cultivated in 2-L Erlenmeyer flasks containing 1 L of medium. After inoculation (10^7 spores/mL), the

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cultures of *P. camemberti* and *P. roqueforti* were incubated on a rotary shaker (100 rpm) at 20 and 28°C, respectively. The concentration of glucose was monitored by a glucose oxidase test (Stanbio Laboratory Inc., San Antonio, TX). After the appropriate time of culture, the biomasses were harvested and recovered by filtration, lyophilized, and stored at –80°C. The dried mycelia were weighed to estimate the microbial growth.

Preparation of crude enzymatic extracts. The lyophilized cells were suspended in sodium phosphate buffer solution (0.01 M, pH 7.0) and homogenized (5 × 15 s, 22,500 rpm) using a Virtis homogenizer (Virtis Company, Gardiner, NY). The cell suspensions were further subjected to glass bead homogenization (2 × 2 min) using a MSK cell homogenizer (Braun, Melsungen, Germany).

The resulting homogenized cell suspensions of *P. camemberti* and *P. roqueforti* were centrifuged (12,000 × *g*, 15 min) and the supernatants were lyophilized, whereas the pellets were discarded. The lyophilized enzymatic extracts were successively defatted with cold (–30°C) acetone and diethyl ether and subjected to DNA precipitation using protamine sulfate, as described previously (6). The resulting extracts from *P. camemberti* and *P. roqueforti* were considered to be the crude enzyme extracts, FI and FI′, respectively.

Partial purification of enzymatic extracts. The partial purification of the LOX crude extracts was performed by ammonium sulfate precipitation at 10–50 and 0–40% of saturation for *P. camemberti* and *P. roqueforti*, respectively. The suspensions were allowed to stand for 30 min and then centrifuged (35,000 × *g*, 15 min). The precipitates, considered as the partially purified enzymatic extracts of *P. camemberti* (FII) and *P. roqueforti* (FII′), were recovered and resuspended in minimal volumes of sodium phosphate buffer solution (0.01 M, pH 7.0) and dialyzed against sodium phosphate buffer solution (0.001 M, pH 7.0) for a period of 12 h. The desalted enzymatic suspensions were lyophilized and used throughout this study for kinetic studies as well as for the characterization of end products.

Protein measurement. The protein concentration of the enzymatic fractions was determined according to a modification of the Lowry method (36). Bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used as a standard for the calibration curve.

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 Nu-Chek-Prep (Elysian, MN). Stock solutions of these substrates were prepared at a concentration of 4.0×10^{-3} M in the selected buffer solution. The substrate buffer mixture was homogenized by adding 0.5% (vol/vol) of polyoxyethylenesorbitan monolaurate (Tween 20).

Enzyme assay. LOX activity was assayed using a preincubated (25°C, 3 min) reaction mixture containing 187.5 µL of a 4×10^{-3} M substrate solution, the enzymatic extract (33 µg of protein), and a sufficient amount of the appropriate buffer solution to adjust the final volume to 1 mL. All experiments were performed in triplicate. A control assay, containing all the components except the enzyme preparation, was run in tandem with these trials. LOX activity was demonstrated by the increase in absorbance at 234 nm (37) using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc., San Raman, CA). The specific activity was defined as nmol of conjugated diene HPOD produced per mg of protein per min, using a molar extinction coefficient of 25,000 M^{-1} cm^{-1} (38).

Effect of pH. The effect of pH on LOX activity was studied using the enzyme assay procedure described above. Citrate phosphate (0.1 M) buffer solution was used for the pH range of 4.0 to 5.5; sodium phosphate (0.1 M) buffer solution for the pH range of 6.0 to 8.0; glycine-NaOH (0.1 M) buffer solution for the pH range of 8.5 to 10.5; phosphate-NaOH (0.1 M) buffer solution for the pH of 11.0 and 11.5; and KCl-NaOH (0.1 M) solution for the pH range of 12.0 to 13.0.

Preparation and determination of standards. The HPOD standards, the 9(*S*)- and 13(*S*)-HPOD, were prepared using tomato and soybean LOX according to the procedures described previously by Matthew *et al.* (39) and Hamberg and Samuelsson (40), respectively.

Recovery of HPOD. The partially purified LOX extracts (10 mg protein) of *P. camemberti* and *P. roqueforti* were incubated with 30 mL of the substrate buffer mixture prepared at the appropriate pH. After 12 min of incubation, the enzymatic reaction was halted by the addition of 4 M HCl (pH 3.0). The HPOD were extracted with diethyl ether, and all traces of protein and Tween-20 were eliminated by extraction with water. The diethyl ether was evaporated using a gentle stream of nitrogen.

HPLC. The HPLC system used for the analyses was Beckman Gold (Beckman Instruments, Fullerton, CA), equipped with an ultraviolet (UV) diode-array detector (DAD) (Beckman, model 168) and a laser light-scattering detector (LLSD) (Varex Corporation, Burtonsville, MD) assembled in series and fitted with a computerized data-handling integrated system (Beckman model 126). A Beckman analog interface model 406 was used to transfer data from the LLSD to the HPLC system. Scanning between 190 and 270 nm was done each second for the entire run; in addition, UV detection was performed specifically at 234 nm. The analyses using the LLSD were performed at 75°C in the presence of an inert gas (N_2) with a flow rate of 40 mL/min. Injection was carried out with an automatic injector (Varian, Autosampler 9095, Varian Associates Inc., Walnut Creek, CA) fitted with a 20-µL loop.

The oxidized fractions produced by the enzymatic extracts were first applied to reversed-phase HPLC and the fractions containing the HPOD were collected, as described previously (31). The standard HPOD as well as the collected HPOD produced by the enzymatic extracts were dissolved in methanol and reduced to the corresponding hydroxides of linoleic acid

(HOD) with the use of $NaBH_4$ (31). The HOD isomers were separated by normal-phase HPLC (NP-HPLC), using a µ-Porasil silica column (300 × 3.9 mm i.d., 10 µm; Waters Corp., Milford, MA); the eluant system was a mixture of hexane/2-propanol/acetic acid (990:10:1, vol/vol/vol) at a flow rate of 1.0 mL/min.

The 9- and 13-HOD isomers, separated by NP-HPLC, were collected and subjected to chiral-phase-HPLC (CP-HPLC) analyses. The stereochemistry of the HOD isomers was analyzed with a Chiralcel OD column (Daicel Chemical Industries, Exton, PA) using a solvent mixture composed of *n*-hexane/2-propanol/acetic acid (1,000:30:1, vol/vol/vol) at a flow rate of 1.0 mL/min. HOD isomers were detected at 234 nm.

Derivatization of HOD. The HOD were derivatized into their corresponding methyl trimethylsilyloxystearate (MTMS) derivatives by successive methylation with diazomethane, hydrogenation with platinum oxide, and trimethylsilylation with *N,N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), as described by Bisakowski *et al.* (31).

Gas–liquid chromatography/mass spectrometry (GC/MS) of derivatized HPOD. The GC/MS system used for the analyses of the derivatized HOD was a 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). 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 HPOD 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 to 250°C, followed by a rate of 10°C/min to a maximum of 290°C where it was held for 3 min. For the GC analysis, the flow rates were set at 30, 400 and 10 mL/min for the hydrogen, air, and makeup gas (He), respectively; however, the carrier gas (He) flow rate was kept at 25 psi. Mass spectra were obtained by electron impact (EI) ionization at 70 eV.

RESULTS AND DISCUSSION

Growth of Penicillium *sp.* During a 16-d period of culture incubation, a kinetic study was carried out that monitored the dry weight and LOX activity of the *Penicillium* sp. as well as the changes in glucose and pH of the culture medium. The crude enzymatic extracts were directly assayed for LOX activity without being defatted or subjected to DNA removal procedures. The results (Fig. 1) show that, after 10 d of incubation, both strains of *Penicillium* oxidized most of the glucose and stopped growing as indicated by the dry weight of the biomasses. The LOX activity from *P. camemberti* remained fairly constant during the incubation of the culture whereas that of *P. roqueforti* slightly increased from 11.8 nmol diene per mg protein per min, after 4 d of culture, to 13.8 nmol diene per mg protein per min after 10 d. The pH of

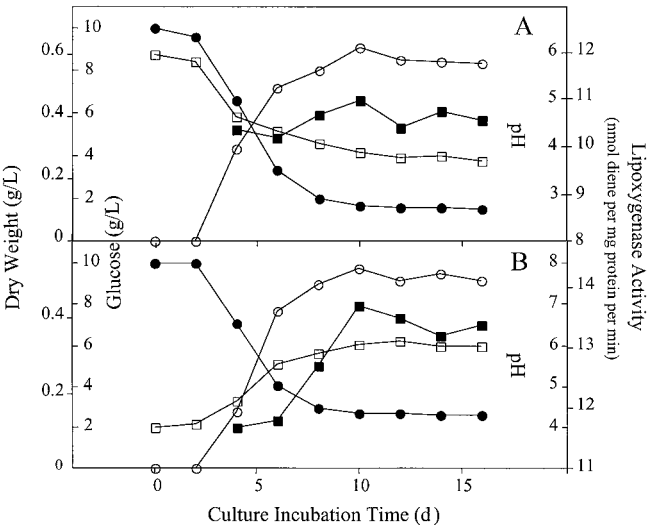

FIG. 1. Changes in dry weight (○), glucose (●), pH (□) and lipoxygenase activity (■) during the culture of (A) *Penicillium camemberti* and (B) *P. roqueforti*.

the culture medium varied mostly during the oxidation of glucose and growth of mycelia. The pH of the culture medium of *P. camemberti* decreased from 6 to 4 whereas that of *P. roqueforti* increased from 4 to 6 during the first 10 d of culture incubation.

The biomasses of both strains were harvested and homogenized after 10 d of culture, corresponding to the maximal dry weight of mycelium as well as the maximal LOX activity.

Partial purification of LOX. The experimental findings (data not shown) indicated that the partial purification of crude enzymatic extracts from *Penicillium* sp., by ammonium sulfate precipitation at 10–50% of saturation for *P. camemberti* and 0–40% of saturation for *P. roqueforti*, resulted in a 2- and 2.3-fold increase in the specific LOX activity, respectively. On the basis of these findings, partially purified fractions from *P. camemberti* (FII) and *P. roqueforti* (FII′) were employed in further studies.

Effect of pH on LOX activity. The effects of pH on the LOX activity of the enzymatic extracts of *P. camemberti* (FII) and *P. roqueforti* (FII′), were determined spectrophotometrically over a wide range of pH values (4.0 to 13.0). The results (Fig. 2) indicate the presence of two pH optima for both LOX extracts. By using linoleic acid as substrate, LOX extracts from *P. camemberti* and *P. roqueforti* displayed a major activity at pH values of 6.5 and 5.5, respectively, and a minor one at pH 8.0.

Herman and Hamberg (7) reported that the partially purified enzyme extract of *Saprolegnia parasitica* exhibited activity over a broad pH optimum with the highest at pH 7.5. Bisakowski *et al.* (35) observed optimal activities at pH 6.0 and 10.5 for the purified LOX fractions from *F. proliferatum*. Similarly, a crude extract from *F. oxysporum* displayed three optima at pH 6.0, 10.0, and over 12.0 (41).

The activity of LOX from *Penicillium* sp. also was determined as a function of pH, using arachidonic acid as sub-

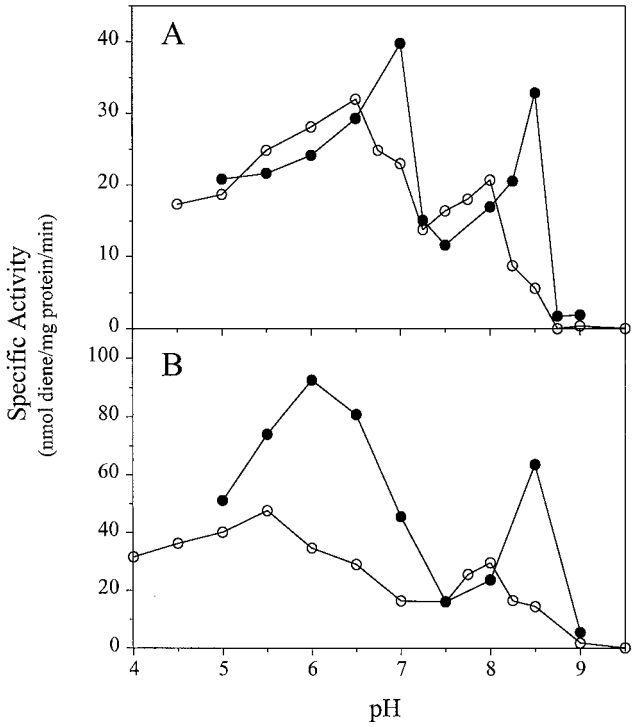

FIG. 2. Effect of pH on the lipoxygenase activity of the partially purified extracts of (A) *Penicillium camemberti* and (B) *P. roqueforti*, using linoleic acid (○) or arachidonic acid (●) as substrates.

strate. The results (Fig. 2) indicate the presence of two pH optima, with a major activity at pH 7.0 for *P. camemberti* and at pH 6.0 for *P. roqueforti*, and a minor one at pH 8.5 for both microorganisms; these pH optima values are slightly higher (0.5 pH unit) than those obtained for linoleic acid. Iny *et al.* (42) reported that LOX from *Thermoactinomyces vulgaris* had two slightly different pH optima, at 6.0 and 6.5, with linoleic and arachidonic acids, respectively.

Substrate specificity studies. The results of substrate specificity studies (Table 1) demonstrate that the enzymatic extracts showed highest activity toward linoleic acid at pH 6.5 and 8.0 for *P. camemberti* and at pH 8.0 for *P. roqueforti*. However, at pH 5.5, the LOX extract from *P. roqueforti* demonstrated a higher specificity for arachidonic and linolenic acids with relative activities of 175 and 122%, respectively. The results obtained for the *P. camemberti* extract are close to those obtained by Shechter and Grossman (15) and Kuo *et al.* (22), who showed that arachidonate and linoleate were more appropriate as substrates than linolenate for the purified LOX fractions of *Saccharomyces cerevisiae* and *Ulva lactuca*, respectively. Iny *et al.* (42) reported a higher activity for the LOX extract of *T. vulgaris*, using linoleic acid as substrate rather than linolenic and arachidonic acids.

The results (Table 1) also indicate that the LOX from *Penicillium* sp. exhibited a preferential specificity toward the free fatty acids and a comparatively lower one for the fatty acid acylglycerols. Similar results were obtained by Bisakowski *et al.* (6,32–34) where the partially purified LOX extracts from *F. oxysporum*, *F. proliferatum*, *S. cerevisiae*, and *Chlorella pyrenoidosa* demonstrated a preference for linoleic acid in comparison to the polyunsaturated fatty acid acylglycerols. Kuo *et al.* (22) also reported a similar observation for the LOX activity of the alga *U. lactuca*. However, the fungus *P. orbiculare* (9) was reported to possess a LOX activity capable of oxidizing, with a similar efficiency, both polyunsaturated fatty acids and triacylglycerols of polyunsaturated fatty acids.

Kinetic studies. The V_{max} and K_m values (Table 2) for the LOX, using linoleic and arachidonic acids as substrates, were calculated from Lineweaver-Burk plots of 1/v vs. 1/[*S*]. The results indicate, with respect to the pH, that lower K_m and higher V_{max} values were determined at the more acidic regions of the two pH optima. In addition, the experimental findings show that lower K_m and higher V_{max} values were obtained with arachidonic acid as substrate rather than with linoleic acid. The ratios of V_{max}/K_m, defined as the enzymatic catalytic efficiency, showed that arachidonic acid was a more appropriate substrate than linoleic acid. Moreover, the LOX extract of *P. roqueforti* displayed a higher catalytic efficiency than that of *P. camemberti*.

The results (Table 2) also indicate that, using linoleic acid as substrate, the K_m values for the LOX activities from both microorganisms are relatively close to that reported in the literature for the purified LOX fraction from the mitochondrial

TABLE 1
Substrate Specificity of Partially Purified Lipoxygenase Extracts from *Penicillium* sp.

	P. camemberti				*P. roqueforti*			
	pH 6.5		pH 8.0		pH 5.5		pH 8.0	
Substrate	Specific activity[a]	Relative activity (%)[b]	Specific activity[a]	Relative activity (%)[b]	Specific activity[a]	Relative activity (%)[b]	Specific activity[a]	Relative activity (%)[b]
Linoleic acid	31.1	100	22.6	100	47.8	100	29.4	100
Linolenic acid	11.6	37	13.7	61	58.1	122	22.9	78
Arachidonic acid	28.1	90	19.2	85	83.7	175	23.4	80
Monolinolein	17.6	57	3.7	16	18.0	38	4.1	14
Dilinolein	12.2	39	4.4	19	16.6	35	4.9	17
Trilinolein	4.4	14	6.0	27	4.9	10	4.3	15

[a]Specific activity was defined as nmol conjugated diene hydroperoxides produced per min per mg protein.
[b]Relative activity was defined as the relative percentage of specific activity obtained with the substrate compared to that with linoleic acid.

TABLE 2
Kinetic Studies of Partially Purified Lipoxygenase Extracts from *Penicillium* sp.

		P. camemberti			*P. roqueforti*		
Substrate	pH	K_m[a]	V_{max}[b]	Catalytic efficiency[c]	K_m[a]	V_{max}[b]	Catalytic efficiency[c]
Linoleic acid	5.5	ND	ND	ND	0.21	55.7	2.67×10^{-4}
	6.5	0.39	46.2	1.19×10^{-4}	ND	ND	ND
	8.0	0.44	33.6	0.76×10^{-4}	0.26	37.7	1.47×10^{-4}
Arachidonic acid	6.0	ND	ND	ND	0.18	102.0	5.83×10^{-4}
	7.0	0.30	54.3	1.50×10^{-4}	ND	ND	ND
	8.5	0.36	44.7	1.23×10^{-4}	0.32	83.6	2.62×10^{-4}

[a]The K_m values were defined as mM of substrate.
[b]The specific activities of V_{max} values were defined as nmol conjugated diene hydroperoxides produced per min per mg protein.
[c]The catalytic efficiency was defined as the ratio of V_{max} to K_m. ND, not determined.

fraction of *S. cerevisiae* (15) and those reported for the purified LOX extracts from *T. vulgaris* and *F. oxysporum* (5,42).

Characterization of LOX end products. Figure 3 shows the NP-HPLC elution profile obtained for the $NaBH_4$-reduced HPOD which had been enzymatically produced by the LOX activity of *P. camemberti* at pH 6.5. Similar elution profiles were obtained for the reduced HPOD produced by the same extract at pH 8.0 as well as by the *P. roqueforti* fraction at pH 5.5 and 8.0. Compounds 1 and 3, with predominant peaks at 17.0 and 27.8 min of elution time, as well as compounds **1′** and **3′** at 24.6 and 32.8 min, were detected with the DAD at 234 nm and the LLSD; similar elution profiles (not shown) of these compounds **1** and **1′**, and **3** and **3′**, were obtained with the 13- and 9-HOD standards, respectively. The results also show the presence of another compound, **2**, at 23.3 min of elution time, in the LLSD analyses.

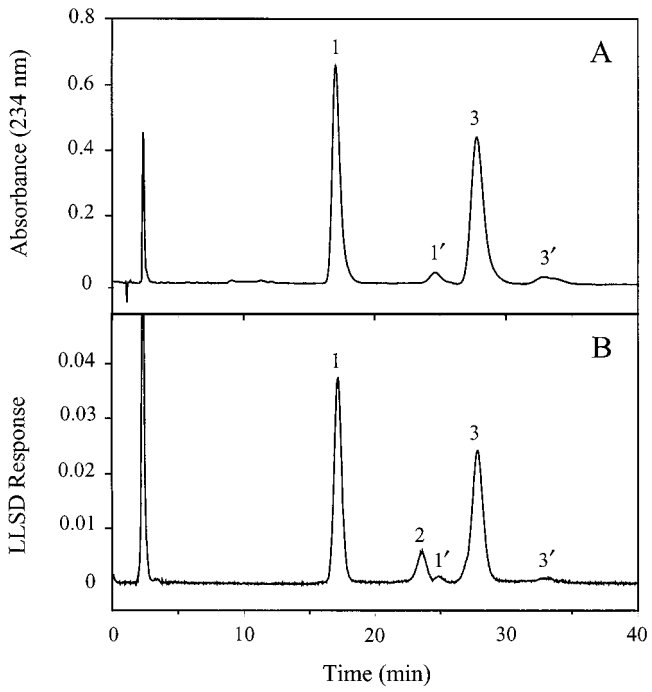

FIG. 3. Sample of normal-phase high-performance liquid chromatographic elution profiles of the reduced hydroperoxides of linoleic acid produced by the enzymatic extract of *Penicillium camemberti*, using (A) ultraviolet-diode-array detection at 234 nm, and (B) laser light-scattering detection (LLSD).

The geometric configuration of the double bonds of compounds **1**, **1′**, **3** and **3′** , detected at 234 nm, was investigated by NP-HPLC analyses coupled with DAD. The UV spectra (Table 3) show that the major compounds, **1** and **3**, possess a maximal wavelength of absorbance (λ_{max}) at 233 nm which is characteristic of *cis,trans* isomers; however, the lower λ_{max} of compounds **1′** and **3′**, hypsochromically shifted to 229 nm, is characteristic of *trans,trans* isomers (43).

The reduced HPOD in the NP-HPLC analyses were collected separately and derivatized into their corresponding MTMS derivatives for GC/MS analyses. The results (Table 3) show that the MTMS derivatives of the collected individual NP-HPLC peaks, **1′**, **2**, **3**, and **3′** each corresponded to only one single GC peak; however, the derivatization of peak **1** to its corresponding MTMS derivative resulted in a major GC peak **1a** and an occasionally minor one **1b**.

The mass spectrum of the MTMS derivatives (Table 3) of compounds **1a** and **1′** showed two intense ions with an *m/z* of 173 and 315, while that of compounds **3** and **3′** had two intense ions of *m/z* 229 and 259, which are characteristic fragmentation patterns of the MTMS derivatives of the 13- and 9-HPOD, respectively (31). The experimental results (not shown) indicated that the MTMS derivatives of the 13- and the 9-HPOD standards possess the same GC elution times and the same mass spectra as the MTMS derivatives of compounds **1** and **1′**, and **3** and **3′**, respectively. The results also show (Table 3) that the mass spectrum of the MTMS derivative of compound **1b** possessed two intense ions of a *m/z* ratio of 187 and 301, and that of compound **2** had two intense ions of a *m/z* ratio of 215 and 273, which are the characteristic fragmentation patterns of the MTMS derivatives of the 12- and 10-HPOD, respectively (31).

Table 4 shows the relative qualitative production of HPOD by the two microbial LOX extracts as determined by the combined HPLC–LLSD and GC/MS analyses. The main reduced 13*c*,*t*-HPOD and the minor reduced 12-HPOD were not resolved by NP-HPLC but their corresponding MTMS derivatives were separated by GC. The results (Table 4) indicate that

TABLE 3
Characterization of Reduced Hydroperoxide Isomers (HOD) and Their Methyl Trimethylsilyloxystearate (MTMS) Derivatives[a]

NP-HPLC analysis of HOD			GC/MS analysis of MTMS derivatives		
Peak number	Retention time (min)	Maximal λ_{max} (nm)	Peak number	MS fragments[b] (*m/z*)	Identification
1	17.0	233	1a	173 (100%), 315 (62%)	13-MTMS[c]
			1b	187 (100%), 301 (75%)	12-MTMS[d]
2	23.3	205	2	215 (95%), 273 (100%)	10-MTMS[d]
1′	24.6	229	1a	173 (100%), 315 (59%)	13-MTMS[c]
3	27.8	233	3	229 (78%), 259 (100%)	9-MTMS[c]
3′	32.8	229	3	229 (80%), 259 (100%)	9-MTMS[c]

[a]These compounds were obtained by the enzymatic activity of lipoxygenase extracts from *Penicillium camemberti* and *P. roqueforti* using linoleic acid as substrate and were determined by normal-phase high-performance liquid chromatography (NP-HPLC) and gas–liquid chromatography/mass spectrometry (GC/MS).
[b]Relative percentage of the intensity of the defined fragment compared to that of the fragment with the highest intensity.
[c]The compound was identified by its retention time and mass spectrum.
[d]The compound was identified by its mass spectrum.

the 9-, 10- and 13-HPOD were produced by the LOX activity of both *Penicillium* sp. at their two respective pH optima, where the 9- and 13-HPOD were the major (90%) end products. At the more acidic pH, the proportion of the 9- and 13-HPOD produced was similar; however, a relatively higher conversion of linoleic acid to the 13-HPOD was observed at pH 8.0 by the LOX activity of the two *Penicillium* sp.

Moreover, the presence of the 10-HPOD was shown by the HPLC–LLSD and GC/MS analyses; the proportion of the 10-HPOD isomer produced by the LOX activity of the enzymatic extracts of *P. camemberti* and *P. roqueforti* was 8 and 9%, respectively, at pH 6.5 and 5.5, while that produced at pH 8.0 (4%) was lower for both LOX extracts. Linoleic acid was also converted to the 12-HPOD at a proportion of 4 and 1% by the enzymatic extracts of *P. camemberti* and *P. roqueforti* at pH 6.5 and 5.5, respectively.

TABLE 4
Relative Percentage of Reduced HOD and Their MTMS Derivatives[a]

		Relative peak area (%)				
		NP-HPLC analysis of HOD[b]			GC/MS analysis of MTMS derivatives[b]	
Microorganism	pH	9	10	12/13	12	13
P. camemberti	6.5	42	8	50	4	46
	8.0	34	4	62	ND	62
P. roqueforti	5.5	44	9	47	1	46
	8.0	35	4	61	ND	61

[a]These compounds were obtained by the enzymatic activity of lipoxygenase extracts from *Penicillium* sp. using linoleic acid as substrate and were determined by NP-HPLC and GC/MS.
[b]Relative percentage of peak area, determined by HPLC-laser light-scattering detection analysis, of the defined HOD isomer (*cis,trans* and/or *trans,trans*) to that of the total isomers of linoleic HOD.
[c]Relative percentage of peak area of the HOD isomer, obtained by GC analysis, to the total peak area of the 12- and 13-HOD, with respect to the relative percent area of the 12/13-HOD obtained by NP-HPLC analysis. ND, not detected. For other abbreviations see Tables 2 and 3.

Matsuda *et al.* (44) reported that at pH 9.0, the conversion of linoleic acid into 9- and 13-HPOD occurred at a ratio of 70 to 30 by the purified LOX from *F. oxysporum*, respectively. However, the proportion of the 13-HPOD produced was higher at pH 12.0 with a ratio of 56 to 44 for the 9- and 13-HPOD, respectively. Similar ratios of conversion of linoleic acid into the 9- and 13-HPOD were also reported for the LOX extract of *S. cerevisiae* (50:50) (15) as well as for the algal LOX fraction of *Oscillatoria* sp. (52:48) (20). Iny *et al.* (18) reported the production of the 9- and 13-HPOD (56:44), mostly as their *cis,trans* isomers, by the LOX activity from *T. vulgaris*.

In addition to the conversion of linoleic acid into the 9- and 13-HPOD, the LOX extract from *F. oxysporum* was shown to produce certain amounts of 10- and 12-HPOD (31). Moreover, Bisakowski *et al.* (31) reported the production of the 10-HPOD by the LOX extract from *C. pyrenoidosa*. The presence of a LOX activity responsible for the production of the 10-HPOD also was suggested in mushroom homogenates, where the 10-HPOD was shown to be further cleaved by a hydroperoxide lyase activity (45) into volatile eight-carbon compounds, mostly 1-octen-3-ol (23,25). Recently, a possible precursor of 1-octen-3-ol was partially identified as a 10-HPOD in a mycelial homogenate of the edible mushroom *Pleurotus pulmonarius* (27). The formation of C_8 compounds, responsible for mushroom-like flavors of mold surface-ripened cheeses, was associated with the oxidation of linoleic and linolenic acids by LOX (28). The overall results suggest that the 10-HPOD produced by *Penicillium* sp. could be an intermediate metabolite in the production of flavor compounds. High levels of 1-octen-3-ol and other C_8 compounds were reported to be produced by the enzymatic activities from *Aspergillus* and *Penicillium* sp. (29,46).

Although the enzymatic activity of LOX from *Penicillium* sp. indicated (Table 4) a preferential positional specificity toward the 9- and 13-HPOD, the results may also suggest the presence of other enzymatic activities such as those catalyzed by a heme protein or a peroxidase-like activity.

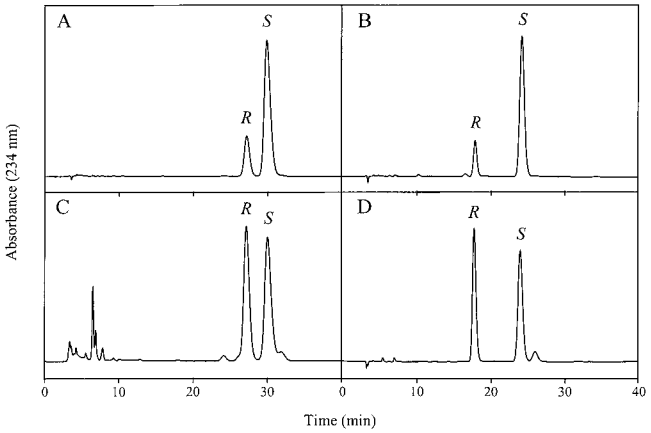

FIG. 4. Sample of chiral-phase high-performance liquid chromatographic elution profiles at 234 nm of reduced hydroperoxide (HPOD) enantiomers (A) 9(*S*)-HPOD standard produced by tomato lipoxygenase (LOX), (B) 13(*S*)-HPOD standard produced by soybean LOX, and (C) 9-HPOD and (D) 13-HPOD produced by the enzymatic extract from *Penicillium camemberti* at pH 6.5.

The analysis of stereospecificity of LOX from *Penicillium* sp. indicated the presence of both (*R*)- and (*S*)-enantiomers. Figure 4 shows samples of the CP-HPLC elution profiles of authentic standards of reduced 9(*S*)-HPOD (Fig. 4A) and 13(*S*)-HPOD (Fig. 4B) as well as those of 9-HPOD (Fig. 4C) and 13-HPOD (Fig. 4D) produced by the LOX extract from *P. camemberti* at pH 6.5. The HPLC analyses of stereospecificity of *P. camemberti* at pH 8.0 as well as those of *P. roqueforti* at pH 5.5 and pH 8.0 were also performed in the same manner. The CP-HPLC profiles of the HPOD standards (Fig. 4A and 4B) indicate that the (*R*)-enantiomer was eluted before the (*S*)-one. Table 5 indicates that an almost racemic mixture was produced by *Penicillium* sp., with a slight excess of the (*S*)-enantiomer, except for the 9-HPOD produced at pH 8.0 by the extract from *P. roqueforti.*

The literature indicated a wide range of LOX preferential stereospecific activity. Mammalian and plant LOX usually catalyze the production of hydroperoxides that have the (*S*)-configuration (3,47). Inversely, an (*R*)-LOX was reported in the culture medium of the fungus *Gäumannomyces graminis* (13) as well as in some of the marine invertebrates (48). However, the literature also reported that other LOX activities exhibited a a wide range of mixtures of (*R*)- and (*S*)-enantiomers, including those of pea seeds LOX-1 (49) and LOX-2 from soybean seeds (50). In addition, Beuerle and Schwab (51) suggested that the formation of (*R*)- and (*S*)-enantiomers of 9- and 13-HOD by apple homogenates was catalyzed by an enzymatic reduction of the corresponding HPOD, obtained originally by a LOX activity. The production of both (*R*)- and (*S*)-enantiomers by the partially purified extracts of *Penicillium* sp. may suggest the presence of LOX isozymes, displaying hence a different product stereospecificity.

TABLE 5
Chiral-Phase High-Performance Liquid Chromatography (CP-HPLC) of Reduced 9- and 13-HOD Isomers[a]

		Relative peak area (%)[b]			
		9-HOD		13-HOD	
Microorganism	pH	(*R*)	(*S*)	(*R*)	(*S*)
P. camemberti	6.5	48.3	52.2	47.2	52.8
	8.0	47.8	52.2	47.0	53.0
P. roqueforti	5.5	47.1	52.9	44.7	55.3
	8.0	51.6	48.4	44.7	55.3

[a]Isomers obtained by the enzymatic activity of lipoxygenase extracts from *Penicillium* sp., using linoleic acid as substrate.
[b]Relative percentage of peak area, determined by CP-HPLC analysis, of the defined HOD enantiomer (*R*) or (*S*) to that of the total of both enantiomers. For abbreviation see Table 3.

ACKNOWLEDGMENTS

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (CORPAQ). The authors would like to thank Dr. E. Spinnler from the INA-Paris, France, for providing the strains of *P. camemberti* and *P. roqueforti*. The authors would also like to thank Ndeye Dioum and Céline Alloyeau for their technical assistance.

REFERENCES

1. Gardner, H.W., Recent Investigations into the Lipoxygenase Pathway of Plants, *Biochim. Biophys. Acta 1084*:221–239 (1991).
2. Siedow, J.N., Plant Lipoxygenase: Structure and Function, *Annu. Rev. Plant Physiol. Plant Mol. Biol. 42*:145–188 (1991).
3. Yamamoto, S., "Enzymatic" Lipid Peroxidation: Reactions of Mammalian Lipoxygenases, *Free Radical Biol. Med. 10*: 149–159 (1991).
4. Mukherjee, J., Studies on Degradation of Fats by Microorganisms. I. Preliminary Investigations on Enzyme Systems Involved in the Spoilage of Fats, *Arch. Biochem. Biophys. 33*:364–376 (1951).
5. Matsuda, Y., T. Satoh, T. Beppu, and K. Arima, Purification and Properties of Co^{2+} Requiring Heme Protein Having Lipoxygenase Activity from *Fusarium oxysporum*, *Agric. Biol. Chem. 40*: 963–976 (1976).
6. Bisakowski, B., S. Kermasha, and M.L. Klopfenstein, Partial Purified Lipoxygenase from *Fusarium oxysporum*: Characterization and Kinetic Studies, *Process Biochem. 30*:261–268 (1995).
7. Herman, R.P., and M. Hamberg, Properties of the Soluble Arachidonic Acid 15-Lipoxygenase and 15-Hydroperoxide Isomerase from the Oomycete *Saprolegnia parasitica*, *Prostaglandins 34*:129–139 (1987).
8. Herman, R.P., and M.M. Luchini, Lipoxygenase Activity in the Oomycete *Saprolegnia* Is Dependent on Environmental Cues and Reproductive Competence, *Exp. Mycol. 13*:372–379 (1989).
9. Nazzaro-Porro, M., S Passi, M. Picardo, R. Mercantini, and A.S. Breathnach, Lipoxygenase Activity of *Pityrosporum in vitro* and *in vivo*, *J. Invest. Dermatol. 87*:108–112 (1986).
10. Simmons, C.A., J.L. Kerwin, and R.K. Washino, Preliminary Characterization of Lipoxygenase from the Entomopathogenic Fungus *Lagenidium giganteum*, in *The Metabolism, Structure, and Function of Plant Lipids*, edited by P.K. Stumpf, J.B. Mudd, and W.D. Nes, Plenum Press, New York, 1987, pp. 421–423.
11. Herman, R.P., M.M. Luchini, and C.A. Herman, Hormone-Dependent Lipoxygenase Activity in *Achlya ambisexualis*, *Exp. Mycol. 13*:95–99 (1989).

12. Herman, R.P., M.M. Luchini, Y.M. Martinez, C.A. Herman, and A.D. Garcia, Cyclic Nucleotides Modulate Lipoxygenase Activity and Reproduction in Oomycetes, *Ibid. 14*:322–330 (1990).
13. Su, C., and E.H. Oliw, Manganese Lipoxygenase: Purification and Characterization, *J. Biol. Chem. 273*:13072–13079 (1998).
14. Perraud, X., S. Kermasha, and B. Bisakowski, Characterization of Lipoxygenase Extract from *Geotrichum candidum*, *Process Biochem. 34*:819–827 (1999).
15. Shechter, G., and S. Grossman, Lipoxygenase from Baker's Yeast: Purification and Properties, *Int. J. Biochem. 15*: 1295–1304 (1983).
16. Lyudnikova, T.A., A.K. Rodopulo, and I.A. Egorov, Lipoxygenase Activity of the Yeast *Saccharomyces vini* Strain Msh-2, *Prikl. Biokhim. Mikrobiol. 20*:175–177 (1984).
17. Shimahara, K., and Y. Hashizume, Properties of a Lipoxygenase-Like Enzyme Produced by *Pseudomonas aeruginosa* Strain A-4, *J. Ferment. Technol. 51*:183–189 (1973).
18. Iny, D., A. Pinsky, M. Cojocoru, and S. Grossman, Lipoxygenase of *Thermoactinomyces vulgaris*, Purification and Characterization of Reaction Products, *Int. J. Biochem. 25*:1313–1323 (1993).
19. Zimmerman, D.C., and B.A.Vick, Lipoxygenase in *Chlorella pyrenoidosa*, *Lipids 8*:264–266 (1973).
20. Beneytout, J.L., R.H. Andrianarison, Z. Rakotoarisoa, and M. Tixier, Properties of a Lipoxygenase in Green Algae (*Oscillatoria* sp.), *Plant Physiol. 91*:367–372 (1989).
21. Kuo, J.M., A. Hwang, H.H. Hsu, and B.S. Pan, Preliminary Identification of Lipoxygenase in Algae (*Enteromorpha intestinalis*) for Aroma Formation, *J. Agric. Food Chem. 44*: 2073–2077 (1996).
22. Kuo, J.M., A. Hwang, and D.B. Yeh, Purification, Substrate Specificity, and Products of a Ca^{2+} Stimulating Lipoxygenase from Sea Algae (*Ulva lactuca*), *Ibid. 45*:2055–2060 (1997).
23. Tressl, R., D. Bahri, and K.H Engel, Formation of Eight-Carbon and Ten-Carbon Components in Mushrooms (*Agaricus campestris*), *Ibid. 30*:89–93 (1982).
24. Grosch, W., and M. Wurzenberger, Enzymic Formation of 1-Octen-3-ol in Mushrooms, in *Progress in Flavour Research 1984*, edited by J. Adda, Elsevier Science Publishers, Amsterdam, 1985, pp. 253–259.
25. Mau, J.L., R.B. Beelman, and G.R. Ziegler, 1-Octen-3-ol in the Cultivated Mushroom, *Agaricus bisporus*, *J. Food Sci. 57*:704–706 (1992).
26. Belinky, P.A., S. Masaphy, D. Levanon, Y. Hadar, and C.G. Dosoretz, Effect of Medium Composition on 1-Octen-3-ol Formation in Submerged Cultures of *Pleurotus pulmonarius*, *Appl. Microbiol. Biotechnol. 40*:629–633 (1994).
27. Assaf, S., Y. Hadar, and C.G. Dosoretz, 1-Octen-3-ol and 13-Hydroperoxylinoleate Are Products of Distinct pathways in the Oxidative Breakdown of Linoleic Acid by *Pleurotus pulmonarius*, *Enzyme Microbiol. Technol. 21*:484–490 (1997).
28. Karahadian, C., D.B. Josephson, and R.C. Lindsay, Contribution of *Penicillium* sp. to the Flavors of Brie and Camembert Cheese, *J. Dairy Sci. 68*:1865–1877 (1985).
29. Spinnler, H.E., O. Grosjean, and I. Bouvier, Effects of Culture Parameters on the Production of Styrene (vinyl benzene) and 1-Octen-3-ol by *Penicillium caseicolum*, *Ibid. 59*:533–541 (1992).
30. Hsieh, R.J., Contribution of Lipoxygenase Pathway to Food Flavors, in *Lipids in Food Flavors*, edited by C.T. Ho and T.G. Hartman, ACS Symposium Series, Washington, DC, 1994, pp. 30–38.
31. Bisakowski, B., X. Perraud, and S. Kermasha, Characterization of Hydroperoxides and Carbonyl Compounds Obtained by Lipoxygenase Extracts of Selected Microorganisms, *Biosci. Biochem. Biotechnol. 61*:1262–1269 (1997).
32. Bisakowski, B., S. Kermasha, V. Lavorel, and J.M. Belin, Partial Characterization of a Lipoxygenase from *Fusarium proliferatum*, *Food Biotechnol. 9*:189–205 (1995).
33. Bisakowski, B., S. Kermasha, and P. Marsot, Partial Purification of Lipoxygenase in *Chlorella pyrenoidosa*, *Biotechnol. Appl. Biochem. 21*:39–48 (1995).
34. Bisakowski, B., S. Kermasha, and C. Schuepp, Partial Purification and Catalytic Properties of Lipoxygenase from *Saccharomyces cerevisiae*, *World J. Microb. Biotechnol. 11*:494–496 (1995).
35. Bisakowski, B., S. Kermasha, and E. Spinnler, Characterization of Purified Lipoxygenase Extracts from *Fusarium proliferatum*, *J. Agric. Food. Chem. 46*:2382–2388 (1998).
36. Hartree, E.P., Determination of Protein: A Modification of the Lowry Method That Gives a Linear Photometric Response, *Anal. Biochem. 48*:422–427 (1972).
37. Surrey, P.K., Spectrophotometric Method for Determination of Lipoxidase Activity, *Plant Physiol. 39*:65–70 (1964).
38. Vick, B.A., A Spectrophotometric Assay for Hydroperoxide Lyase, *Lipids 26*:315–320 (1991).
39. Matthew, J.A., H.W.S. Chan, and T.A. Galliard, A Simple Method for the Preparation of Pure 9-*D*-Hydroperoxide of Linoleic Acid and Methyl Linoleate Based on the Positional Specificity of Lipoxygenase in Tomato Fruit, *Lipids*, *12*:324–326 (1977).
40. Hamberg, M., and B. Samuelsson, On the Specificity of the Oxygenation of Unsaturated Fatty Acids Catalyzed by Soybean Lipoxidase, *J. Biol. Chem. 25*:5329–5335 (1967).
41. Satoh, T., Y. Matsuda, M. Takashio, K. Satoh, T. Beppu, T., and K. Arima, Isolation of Lipoxygenase-Like Enzyme from *Fusarium oxysporum*, *Agric. Biol. Chem. 40*:953–961 (1976).
42. Iny, D., S. Grossman, and A. Pinsky, Lipoxygenase of Thermophilic Bacteria *Thermoactinomyces vulgaris*, Properties and Study on the Active Site. *Int. J. Biochem. 25*:1325–1330 (1993).
43. Toschi, T.G., F. Stante, P. Capella, and G. Lercker, Study on Position and Geometric Configuration of Methyl Linoleate Hydroperoxide Isomers Obtained by Thermo-Oxidation:Chromatographic Analyses of Their Corresponding Hydroxy Derivatives, *J. High. Resolut. Chromatogr. 18*:764–766 (1995).
44. Matsuda, Y., T. Beppu, and K. Arima, Crystallization and Positional Specificity of Hydroperoxidation of *Fusarium* Lipoxygenase, *Biochim. Biophys. Acta 530*:439–450 (1978).
45. Wurzenberger, M., and W. Grosh, The Formation of 1-Octen-3-ol from the 10-Hydroperoxide Isomer of Linoleic Acid by a Hydroperoxide Lyase in Mushrooms (*Psalliota bispora*), *Ibid. 794*:25–30 (1984).
46. Kaminsky, E., S. Stawicki, and E. Wasowicz, Volatile Flavor Compounds Produced by Molds of *Aspergillus*, *Penicillium*, and Fungi imperfecti, *Appl. Microbiol. 27*:1001–1004 (1974).
47. Gardner, H.W., Lipoxygenase as a Versatile Biocatalyst, *J. Am. Oil Chem. Soc. 73*:1347–1357 (1996).
48. Hamberg, M., C. Su, and E. Oliw, Discovery of a Bis-Allylic Hydroperoxide as Product and Intermediate in a Lipoxygenase Reaction, *J. Biol. Chem. 276*:13080–13088 (1998).
49. Kühn, H., R. Wiesner, V.Z. Lankin, A. Nekrasov, L. Alder, and T. Schewe, Analysis of the Stereochemistry of Lipoxygenase-Derived Hydroxypolyenoic Fatty Acids by Means of Chiral Phase High-Pressure Liquid Chromatography, *Anal. Biochem. 160*:24–34 (1987).
50. van Os, C.P.A., G.P.M. Rijke-Schilder, and J.F.G. Vliegenthart, 9-L_R-Linoleyl Hydroperoxide, a Novel Product from the Oxygenation of Linoleic Acid by Type-2 Lipoxygenases from Soybeans and Peas, *Biochim. Biophys. Acta*, *575*:479–484 (1979).
51. Beuerle, T., and W. Schwab, Metabolic Profile of Linoleic Acid in Stored Apples: Formation of 13(*R*)-Hydroxy-9(*Z*),11(*E*)-octadecadienoic Acid, *Lipids 34*:375–380 (1999).

[Received August 6, 1999; accepted December 8, 1999]