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Purification and Partial Characterization of Lipoxygenase from Desert Truffle (*Terfezia claveryi* Chatin) Ascocarps

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A lipoxygenase from *Terfezia claveryi* Chatin ascocarp, a mycorrhizal hypogeous fungus, is described for the first time. The higher proportion of PUFA in *T. claveryi* ascocarps makes lipid rancidity the main factor limiting its storage life. Thus, the studies on LOX from *T. claveryi* are important because this enzyme, among other roles, may be involved in an alteration of lipids leading to consumer rejection. The enzyme has been purified to apparent homogeneity by phase partitioning in the presence of Triton X-114, followed by two steps of cation-exchange chromatography. The purified *T. claveryi* LOX preparation consisted of a single major band with an apparent molecular mass of 66 kDa after sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The enzymic activity exhibited a strong specificity toward linoleic and linolenic acids as substrates, while only 32% activity was observed using γ-linolenic acid. The pH optimum of this enzyme was pH 7.0. When the enzyme reacted with linoleic acid, it produced a single peak, which comigrated with standard 13-hydroperoxy-octadecadienoic acid; 13-hydroperoxy-octadecatrienoic acid was produced during the reaction with linolenic acid.

KEYWORDS: Ascocarp; desert truffle; mycorrhizal hypogeous fungi; lipoxygenase; *Terfezia claveryi*; Triton X-114; HPOD

INTRODUCTION

Desert truffles or “turmas” are a complex family of mycorrhizal hypogeous fungi, containing species of the genera *Balsamia*, *Picoa*, *Terfezia*, *Tirmania*, and *Tuber*, and are found in many Mediterranean countries (*1*). Desert truffles are of considerable interest for ecological, agroforestry, and commercial purposes. Moreover, there is growing interest in introducing desert truffle cultivation into dry environments as a useful way of exploiting lands which, until now, have been regarded as unproductive (*2*). *Terfezia claveryi* Chatin is a common hypogeous ascomycete in marl-gypsum soils of semiarid areas, which establishes mycorrhizal symbiosis with several annual and perennial species of the *Helianthemum* genus (*1*, *2*). Its complex life cycle involves a first phase of symbiotic association of the fungal hyphae with the root and finally the organization of a hypogeous fruitbody with asci and ascocarps.

Many studies have reported that diets with a high unsaturated fatty acid to saturated fatty acid ratio (UFA/SFA) might reduce the risk of coronary heart disease by inducing changes in serum lipids, including cholesterol (*3*). Some authors have also demonstrated the ability of some edible fungi to reduce blood serum cholesterol (*4*). Desert truffles are healthy foods rich in fiber, proteins, vitamins, and minerals (*5*, *6*). In *T. claveryi*, the UFA/SFA ratio showed values greater than unity and the polyunsaturated/monounsaturated fatty acid (PUFA/MUFA) ratio reached a value of 5.0 (*6*). The higher proportion of PUFA, more than 50% of total fatty acids (*6*), makes lipid rancidity the main factor limiting storage life, because lipid peroxidation gives rise to unpleasant odors and tastes, with the concomitant loss of sensory quality in food, leading to consumer rejection.

Lipoxygenases (linoleate: oxygen oxidoreductase, EC 1.13.11.12) are nonheme iron-containing enzymes that use molecular oxygen in the dioxygenation of a fatty acid containing one or more 1,4-*Z*,*Z*-pentadiene systems. LOX may be involved in the development of oxidative rancidity in foods (*7*), but this enzyme may also contribute to flavor formation in some plant foods (*8*).

LOX is present in a wide variety of plant and animal tissues. Some plant LOX are constitutive, whereas others are expressed by wounding and by fungal pathogens (*9*). However, there is little information on fungal LOX and on its physiological role. The presence of this enzyme has been reported in molds such as *Fusarium* (*10*), *Geotrichum candidum* (*11*), *Penicillium* (*12*), *Mortierella* (*13*), and *Aspergillus niger* (*14*) and in the mushroom *Morchella esculenta* (*15*). However, until now, the only edible mushroom from which LOX has been purified to homogeneity is *Pleurotus ostreatus* (*16*). In addition, LOX activity has been reported as being responsible for the bioconversion of linoleic acid (LA) into 10-HPOD, which is, in turn,

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enzymatically cleaved to produce octenol (*17*, *18*), the most important aroma compounds in various species of mushrooms. However, these pathways are not completely clear, and the LOX that produces 10-HPOD from linoleic acid has not yet been found in nature.

The present work is part of our ongoing research in this laboratory on the influence of different enzymes on the quality of desert truffles (*19*−*21*) and reports for the first time the presence of LOX in *T. claveryi* ascocarps. The enzyme has been purified to homogeneity, and some of its properties have been examined.

MATERIALS AND METHODS

Fungal Material. Ascocarps of *T. claveryi* were collected in Zarzadilla de Totana (Lorca, Murcia, Spain), where they were associated with *Helianthemum almeriense* Pau shrubs and used a few hours after collection or after storage at −20 °C.

Reagents. Bicinchoninic acid, bovine serum albumin (BSA), Brilliant Blue R, high-molecular-mass marker kit (SDS-6H), copper sulfate pentahydrate 4%, "Infinity" triglycerides reagent kit, and SDS were purchased from Sigma (Madrid, Spain). Triton X-114 (TX-114) and linoleic (LA), linolenic (LN), and γ-linolenic acids were obtained from Fluka (Madrid, Spain). The remaining reagents were of analytical grade.

Enzyme Purification. Pieces of *T. claveryi* ascocarps, containing both peridium and gleba, were suspended in 0.1 M sodium phosphate at pH 7.0 in a ratio of 1:5 (w/v) before being homogenized with a mortar and pestle at 4 °C. The homogenate was then centrifuged at 15000*g* for 20 min at 4 °C. This supernatant was subjected to temperature phase partitioning by adding 20% TX-114 (w/v) at 4 °C, so that the final detergent concentration was 8% (w/v). The mixture was kept at 4 °C for 5 min and then warmed to 37 °C in a thermostatic bath. After approximately 18 min, the solution became spontaneously turbid, because of the formation, aggregation, and precipitation of large mixed micelles of detergent that contained lipids, hydrophobic proteins, and phenolic compounds. This solution was centrifuged at 15000*g* for 15 min at 30 °C. The detergent-rich phase was discarded, and the clear detergent-poor supernatant was subjected to a second temperature phase partitioning by adding 20% TX-114 (w/v) at 4 °C, so that the final detergent concentration was 6%. After centrifugation (15000*g*, 15 min, 30 °C), the supernatant was either used immediately or stored at −80 °C (where it was stable for more than 5 months). The buffer of the phase-partitioning supernatant was changed to 50 mM sodium phosphate at pH 5.5 by ultrafiltration (Amicon Ultra 10 000 MWCO). Aliquots of this extract were loaded onto a 1 mL Resource S column connected to an Äkta purifier (Amersham-Pharmacia Biotech, Barcelona, Spain) and equilibrated with 50 mM sodium phosphate buffer at pH 5.5 at 1 mL/min. LOX activity was eluted from the column with a NaCl gradient from 0 to 1 M NaCl. The separation was followed at 280 nm. Column fractions were assayed routinely in a reaction medium containing 0.1 M sodium borate buffer at pH 10.0 and 180 μM linoleic acid. The reaction was started by adding the enzyme solution, and the increase in absorbance at 234 nm ($\epsilon = 25\,000$ M^{-1} cm^{-1}) was followed at room temperature in a Uvikon-940 spectrophotometer (Kontron AG, Switzerland). One enzymatic unit (U) is defined as the amount of enzyme that gives rise to the formation of 1 μmol of conjugated diene/min in this reaction medium. Samples containing LOX were stabilized by adding TX-100 (final concentration of 0.04%, v/v). Aliquots containing LOX activity were mixed, loaded in a Resource S (1 mL) column, and equilibrated with 50 mM sodium phosphate buffer at pH 5.5 containing 0.04% TX-100. The elution buffer consisted of 1 M NaCl in 50 mM sodium phosphate at pH 5.5.

Protein Determination. The protein content was measured by the bicinchoninic acid method (*22*) using BSA as the standard. Protein concentrations in the chromatographic fractions were determined by measuring the absorbance at 280 nm and multiplying by 0.71 to give the protein concentration in mg/mL (*23*).

Determination of Triglyceride Concentration. Triglycerides were determined using the "Infinity triglycerides reagent kit" from Sigma, as specified by the supplier.

Enzyme Activity Assays. *Spectrophotometric Determination of LOX Activity.* The optimum temperature was determined spectrophotometrically at 234 nm with an HP 8452A (Hewlett−Packard) diode array spectrophotometer. The temperature of the reaction medium (18 μM LA in 0.1 M sodium phosphate buffer at pH 7.0) was adjusted by using a HP89090A Peltier accessory (Hewlett−Packard) before starting the reaction with the enzyme.

Polarographic Determination of LOX. Substrate specificity and the pH optimum were determined by measuring oxygen consumption with a Clark-type electrode (Hansatech Ltd., Norfolk, U.K.). The reaction medium used to calculate the pH optimum was 10 μL of 100 mM LA in ethanol, 25 μL of purified LOX, and 965 μL of one of the following buffers (0.1 M): sodium acetate (pH 4.0), sodium phosphate (pH 5.0, 5.5, 6.0, 6.5, 7.0, and 7.6), or sodium borate (pH 7.6, 8.4, 9.0, and 10.0). To determine the substrate specificity, 1-mL samples consisting of 180 μM substrate in 0.1 M phosphate buffer at pH 7.0 were vigorously shaken before use so that they would become air-saturated. They were then transferred to the stirred, thermostated oxygraph chamber (Hansatech Ltd., Norfolk, U.K.), where the reaction was started by adding the enzyme. The oxygraph was calibrated using the method proposed by Rodríguez-López et al. (*24*).

The experiments were performed in triplicate and the mean and standard deviation were plotted.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of LOX extracts in the presence of 2-mercaptoethanol was on 10% acrylamide gels according to the method of Laemmli (*25*). The power condition was 200 V, constant voltage setting. Chromatographed samples were concentrated by ultrafiltration (Amicon Ultra 10 000 MWCO). Gels were internally calibrated using a high-molecular-mass marker kit (Sigma SDS-6H) containing myosin (205 kDa), β-galactosidase (116 kDa), phospholipase b (97.4 kDa), BSA (66 kDa), albumin egg (45 kDa), and carbonic anhydrase (29 kDa). The gels were stained for protein visualization with Coomassie Brilliant Blue.

Analysis of LOX Products. For product analysis, 30 mL of a 96 μM solution of LA or LN acids in 0.1 M borate buffer (pH 10.0) or 0.1 M phosphate buffer (pH 7.0) was incubated with 500 μL of enzyme extract under constant aeration. A sample of the incubation medium was placed in a cuvette, and the reaction was followed spectrophotometrically at 234 nm. When the reaction was completed, the reaction medium was acidified to pH 4.0 with HCl, and the products were extracted with an octadecyl solid-phase extraction column eluted with methanol. Methanol was evaporated with a stream of N_2, and the products were resuspended in hexane/2-propanol/AcH (100:1.6:0.1). Samples were injected using a Rheodyne 7125 loop injector (20 μL) and analyzed with a Kontron-420 HPLC (Kontron AG, Switzerland) on a ChromSpher Si column (250 × 4.6 mm), with a 5 μm typical particle size. Products were eluted isocratically with hexane/2-propanol/AcH (100:1.6:0.1, v/v/v) at a flow rate of 1 mL/min and detected at 234 nm using a Kontron 430 UV detector (*26*). The products of *T. claveryi* LOX were identified by HPLC, comparing their retention time with standards of 13- and 9-HPOD or 13- and 9-HPOT or by co-injecting the samples with those standards. 13-HPOD and 13-HPOT were obtained by the reaction of LA and LN with soybean LOX in 0.1 M sodium borate buffer at pH 10.0, while 9-HPOD and 9-HPOT were the products of the same acids with potato LOX (*27*) in sodium phosphate buffer at pH 6.3. These compounds were extracted using the procedure described above, flushed with N_2, and stored at −20 °C.

RESULTS AND DISCUSSION

Purification. Enzyme purification in fungus extracts is difficult because of the large variety and quantity of secondary products that can bind tightly to the enzymes and change their characteristics (*28*). To overcome this, different methods have been developed, including acetone powders, ammonium-sulfate fractionation, salts, insoluble polymers, and detergents. Among the latter, TX-114 shows the special feature of forming clear solutions in buffers at 4 °C, while it separates into two phases at 25 °C because of the formation of large micellar aggregates

Table 1. Purification of Lipoxygenase from *T. claveryi* Ascocarps

purification step	volume (mL)	total activity (units)	total protein (mg)	specific activity (units/mg)	purification (fold)	recovery (%)	total TAG[a] (mg)
crude extract	15	16.5	45.1	0.36	1	100	80
8% TX-114 supernatant	14	15.9	21	0.76	2.1	96.4	36.8
6% TX-114 supernatant	10	11.3	13.6	0.8	2.3	68.5	26.7
1st chrom. RS 1 mL	40	12.4	0.67	18.7	52.1	76.1	ND[b]
2nd chrom. RS 1 mL	133	0.66	ND	ND	ND	4	ND

[a] Triglycerides. [b] ND = not detected.

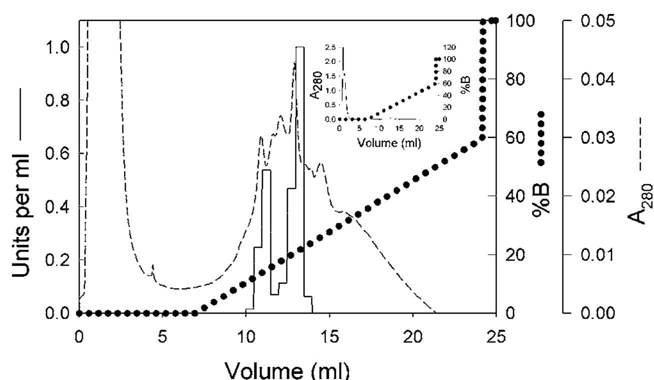

Figure 1. Purification of LOX from *T. claveryi* ascocarps. A total of 1 mL of TX-114 supernatant was loaded on a Resource-S column (1 mL) and eluted with a gradient from 0 to 1 M NaCl in 50 mM sodium phosphate buffer at pH 5.5. The eluate was monitored at 280 nm and collected for the assay of LOX activity as described in the Material and Methods.

(*29*). This characteristic has been used to separate integral proteins from hydrophilic proteins, because the former remains in the detergent-rich phase (*30*). Phase partitioning with TX-114 is a soft method to extract plant and fungal proteins and permits the elimination of lipids (*19*), chlorophylls (*20*), phenols (*19*, *32*), etc. and to obtain a discrete degree of purification (*32*). After the two-phase partitions, a 2.3-fold increase in purification and a recovery of 68.5% was achieved (**Table 1**). This is slightly higher than that reported by Perraud et al. (*11*) for the purification of LOX from *G. candidum* using ammonium sulfate precipitation. After the first chromatographic step on a Resource-S column (**Figure 1**), most of the protein was eliminated (inset of **Figure 1**) and LOX was purified 52 times to obtain a specific activity of 18.7 units/mg of protein and a recovery of 76.1% (**Table 1**). TAG could not be detected in the eluate. The recovery of the purified enzyme was higher than in the previous step, a result similar to that observed by Suurmeijer et al. (*33*) during the purification of soluble tomato LOX. This increase in activity could have been caused by the removal of a LOX inhibitor during this step. In fact, among the constituents of truffles are compounds with antioxidant and antiradical properties, such as vitamins A and C and β-carotene (*5*), some of which have been reported as LOX inhibitors (*34*, *35*). In addition, truffles contain many phenolic compounds (*5*, *19*) that possess a well-known capacity to reduce and chelate ferric iron and to inhibit lipoxygenase (*36*, *37*). On the other hand, the enzyme recovered after the first chromatographic step is very unstable, probably because most of the components of the extraction medium have been eliminated. Different agents were assayed to avoid LOX inactivation. The best results were obtained when TX-100 was added to the enzyme (final concentration of 0.04%, v/v). Under these conditions the enzyme was quite stable at −80 °C and did not lose its activity during a 3-month period. Previously, TX-100 has been proven useful for stabilizing LOX from eggplant chloroplasts (*31*).

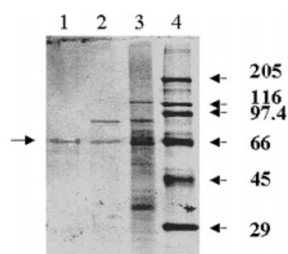

Figure 2. SDS−PAGE (10%) of purified desert truffle LOX. Lane 1, LOX eluted in the second chromatographic run. Lane 2, LOX eluted in the first chromatography run. Lane 3, supernatant of TX-114 (second phase partition). Lane 4, SDS molecular mass markers with mass indicated in kilodaltons. The gel was Coomassie-stained.

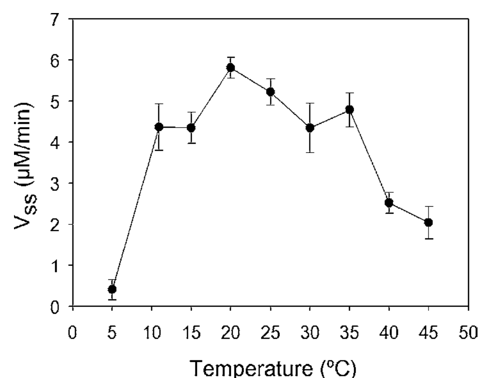

Figure 3. Effect of temperature on the purified LOX activity. The reaction medium containing 18 μM LA in 0.1 M sodium phosphate buffer was adjusted to the desired temperature using a Peltier accessory before starting the reaction by adding the enzyme. The reaction was followed at 234 nm.

Because the enzyme obtained in the first chromatographic step was not homogeneous (**Figure 2**), the active fractions were mixed and loaded on the Resource-S column (1 mL) equilibrated with 50 mM sodium phosphate buffer at pH 5.5 containing 0.04% TX-100. One disadvantage of using TX-100 was that the protein concentration of the LOX-containing fractions could not be calculated because of the strong absorbance that TX-100 showed in the UV region. The percentage of recovery after this last step was very low (4%). During the different purification steps of LOX from *P. ostreatus*, Kuribayashi et al. (*16*) attributed the low recovery of enzyme to the oxidation of thiol groups. In the present paper, the percentages of recovery were high except during the last step, which was necessary to purify LOX to apparent homogeneity (**Figure 2**), although the reason for this loss of enzyme activity is unknown. The SDS−PAGE of this fraction revealed a single band (**Figure 2**) with a molecular weight of 66 kDa, identical to the molecular weight of *P. ostreatus* LOX (*16*).

Although most plant LOXs are predominantly cytosolic soluble enzymes, others have been demonstrated in vacuoles,

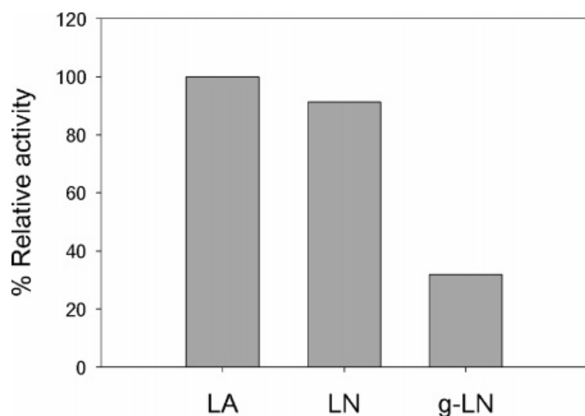

Figure 4. Substrate specificity of LOX from *T. claveryi*. The activity was assayed by polarographic procedures with a reaction medium consisting of 180 μM LA, LN, or γ-LN in 0.1 M phosphate buffer at pH 7.0.

mitochondria, chloroplasts, and the plasma membrane (*31*, *38*−*40*). The localization of fungal LOX remains less studied. Husson et al. (*41*) suggested that lipoxygenase from *F. proliferatum* was mainly distributed among microsomal and mitochondrial fractions. In the present paper, the fact that neither the addition of detergent nor sonication of the extract was needed in the extraction buffer and the partitioning of the enzyme in the aqueous phase suggested that LOX from *T. claveryi* ascocarps was a soluble enzyme.

Effect of pH and Temperature. The pH profile obtained presents the maximum activity at pH 7.0 (data not shown) and is typical of other LOXs such as those from ungerminated barley (*42*), eggplant fruit (*43*), and *Gäumannomyces graminis* lipoxygenase (*44*). The optimum temperature of purified LOX was estimated to be 20 °C (**Figure 3**). The enzyme retained about 75% of its maximum activity at temperatures between 10 and 35 °C, a behavior which is similar to that observed for LOX from *P. ostreatus* (*16*), which retained about 60% of its maximum activity at 10 °C.

Substrate Specificity. When the substrate specificity of the purified LOX enzyme was investigated (**Figure 4**), the highest relative enzymic activity was obtained using linoleic acid (100%) as the substrate, followed by linolenic acid (91%). The lowest relative LOX activity (32%) was exhibited toward γ-linolenic acid. This profile of substrate specificity is similar to that reported for LOX from *P. ostreatus* (*16*). The lipid content of raw truffles was reported to be 69.5 g/kg (dry matter) (*6*). Linoleic acid represents 45.4% of total fatty acids, while linolenic acid represents 5.8% (*6*). These data suggest that endogenous linoleic acid is the preferred substrate for LOX from *T. claveryi*. This profile of substrate specificity is similar to that reported for LOX from *P. ostreatus* (*16*).

Analysis of Reaction Products. It is well-known that the product specificity of soybean LOX-1 depends on the pH of the reaction medium (*45*). Different conversion ratios of linoleic acid into 9- and 13-HPOD at different pH values were reported for LOX from *Fusarium* (*10*), although the presence of different isozymes could not be discounted. Thus, the specificity of purified LOX was characterized using LA and LN at both pH 7.0 and 10.0, with the products formed being analyzed by straight-phase HPLC (**Figure 5**). The elution profile of the products obtained by incubating LA with LOX at the above pH values showed one major peak with a retention time of 12 min (parts **A** and **B** of **Figure 5**). A comparison of the retention times with the standards revealed that in both assays the major peak corresponded to 13-*Z*,*E*-HPOD. The incubation of purified LOX with LN, on the other hand, produced 13-*Z*,*E*-HPOT (>94%) (parts **C** and **D** of **Figure 5**). The correspondence of retention times between the reaction products and the standards was confirmed by co-injecting samples and standards (data not

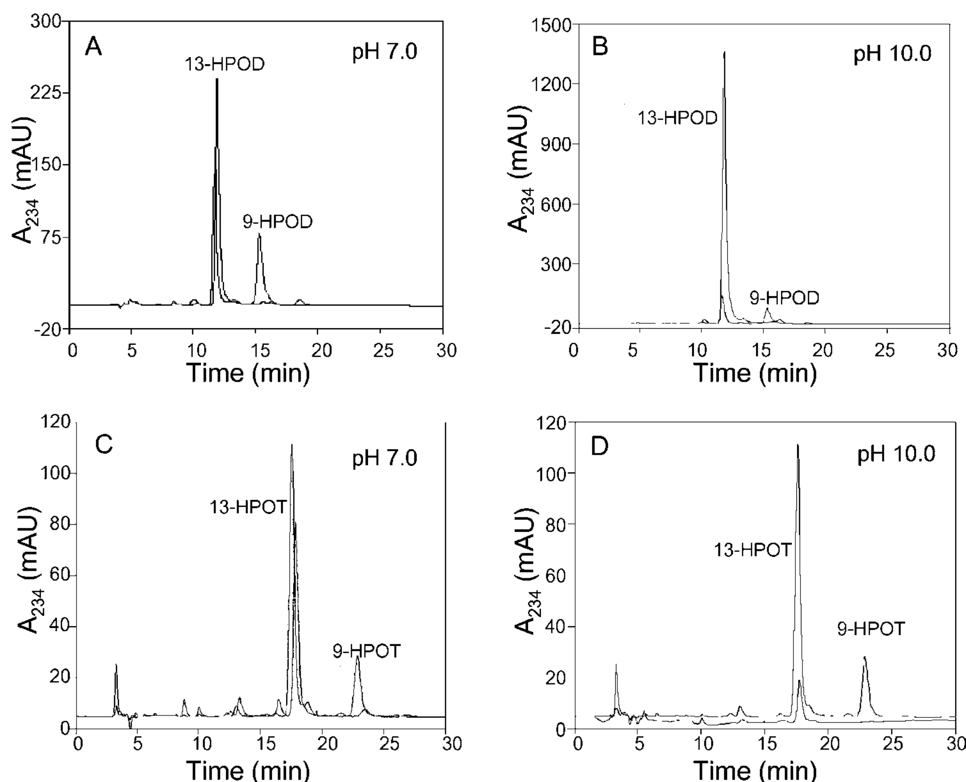

Figure 5. Analysis of reaction products formed by purified LOX from *T. claveryi* from LA (A and B) and LN (C and D). The incubation buffers were 0.1 M sodium phosphate at pH 7.0 (A and C) or 0.1 M sodium borate at pH 10.0. The analyses were carried out on a ChromSpher Si column (250 × 4.6 mm), and the eluent system was hexane/2-propanol/AcH (100:1.6:0.1, v/v/v). mAU = milliabsorbance units.

shown). The UV spectrum of these peaks was typical of conjugated dienes (λ_{max} = 234 nm). Although there are not many studies on fungal LOX products, it has been shown that *Gäumannomyces graminis* (*44*) and *P. ostreatus* (*16*) also produce 13-HPOD as the main reaction product, while other fungi such as *G. candidum* (*11*), *F. proliferatum*, (*10*), or *A. niger* (*14*) synthesize a mixture of 13- and 9-HPOD in different percentages. According to Brash (*46*), the production of a mixture of hydroperoxy products often accompanies the induction of a series of programmed structural changes in the cell, while the synthesis of a single specific hydroperoxide from free fatty acid substrates is related to the formation of biological mediators or signaling molecules. In plants, these hydroperoxides serve as a substrate for enzymes such as hydroperoxide lyase, peroxygenase, hydroperoxide reductase, divinyl ether synthase, or allene oxide synthase (*47*). Some of these PUFA derivatives represent biological signals, which do not require the prior activation of genes (*48*). Although many authors have reported the induction of plant LOXs by fungi (*49*), the role of fungal LOXs in mycorrhizal symbiosis has never been studied.

In conclusion, this paper reports for the first time the purification and product characterization of a LOX from a mycorrhizic fungus. The results obtained indicate that LOX from *T. claveryi* ascocarps is a soluble enzyme with a molecular weight of 66 kDa, which displays its maximum activity at pH 7.0, 20 °C, and using linoleic acid as a substrate. The products of this enzyme using LA or LN as substrates were the 13-hydroperoxides of these fatty acids, regardless of the pH of the reaction medium.

ABBREVIATIONS USED

AcH, acetic acid; BSA, bovine serum albumin; HPOD, hydroperoxy octadecadienoic acid; LA, linoleic acid; LN, linolenic acid; LOX, lipoxygenase; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; UFA, unsaturated fatty acid; SFA, saturated fatty acid; TAG, triglycerides.

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