Characterization and Histochemical Localization of Nonspecific Esterase from Ascocarps of Desert Truffle (*Terfezia claveryi* Chatin)

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An esterase activity from *Terfezia claveryi* Chatin ascocarps, a mycorrhizal hypogeous fungus, is described for the first time. The enzyme was partially purified using phase partitioning in Triton X-114 (TX-114), achieving a reduction of 87% in the triglyceride content and the removal of 63% of phenols. The enzyme showed maximum activity toward short-chain *p*-nitrophenyl esters, and no interfacial activation was observed, indicating that the enzyme responsible for this activity is an esterase and not a lipase. This esterase presented its maximum activity at pH 7.4 and 60 °C. The values obtained for K_m at pH 7.4 were 0.3 mM for *p*-nitrophenyl butyrate and 0.6 mM for *p*-nitrophenyl acetate with catalytic efficiencies (V_{max}/K_m) of 0.23 and 0.32, respectively. *T. claveryi* esterase was inhibited by phenylboric acid, indicating that serine residues were involved in the enzyme activity. This activity was localized only in the hypothecium and was absent from the peridium and gleba.

KEYWORDS: Ascocarp; desert truffle; esterase; localization; mycorrhizal hypogeous fungi; *Terfezia claveryi*; TX-114

INTRODUCTION

Esterases (EC 3.1.1) represent a diverse group of hydrolases widely distributed in animals, plants, and microorganisms. Esterases are usually screened by using chromophoric substances (e.g., *p*-nitrophenyl esters). They are arbitrarily classified as enzymes that hydrolyze substrates in solution, whereas lipases hydrolyze substrates in emulsion (*1*). However, there is no clear-cut distinction between them, and so knowledge of their respective properties is vital to understanding their functions.

Depending on the environmental conditions, esterases can hydrolyze or synthesize fatty acid esters of differing acyl chain lengths. Many of them show a wide substrate tolerance, which has led to the assumption that they have evolved to enable access to carbon sources or to be involved in catabolic pathways. Several authors have observed that mycorrhizal colonization causes an increase in the activity of different enzymes in roots, among them esterase (*2*, *3*). A similar effect was observed in the rhizosphere of maize plants inoculated with arbuscular mycorrhizal fungi (*4*). The increase in the levels of this enzyme has been taken as an indication of the enhanced metabolic activity produced during the establishment of arbuscular mycorrhizal associations (*2*). In addition, the esterase isozyme pattern has been used as a tool for the characterization of arbuscular mycorrhizal fungi (*5*, *6*) and in taxonomic studies of edible mushrooms (*7*−*9*). Esterase has been detected in pathogenous fungi, such as *Pestalotia malicola* (*10*) and *Botrytis elliptica* (*11*), where this enzyme could be involved in the infection processes (*12*−*14*).

The products of the esterase reaction, both short-chain fatty acids and esters, constitute a well-known class of aromatic molecules in foods (*15*−*17*). For example, esterase from the brewer's yeast *Saccharomyces carlsbergensis* was found to contribute to the aroma and flavor of beer (*18*), whereas esterases and lipases produced by fungi such as *Penicillium* species (*17*) play an important role in the flavor of cheese. However, to the best of our knowledge, there are no reports on the catalytic properties of nonspecific esterase in edible mushrooms, despite the role that this enzyme may play in their flavor.

Desert truffles are foods rich in fiber, proteins, vitamins, and minerals (*19*, *20*). They 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 (*21*, *22*). The most important desert truffles are those included in the genera *Terfezia* and *Balsamia*, because of their highly appreciated edible and commercial values. Most studies of truffles have been morphological or bromatological (*19*, *20*), but very few biochemical investigations into their metabolism have been performed.

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In this paper, the presence of esterase in *Terfezia claveryi* is reported for the first time. The enzyme has been partially purified using phase partitioning in Triton X-114 (TX-114), histochemically localized within the ascocarp, and kinetically characterized.

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), chlorogenic acid, copper sulfate pentahydrate 4%, Folin reagent, and an "Infinity" triglycerides reagent kit were purchased from Sigma (Madrid, Spain). Triton X-114 and Triton X-100 were obtained from Fluka (Madrid, Spain). *p*-Nitrophenyl palmitate (pNPP), *p*-nitrophenyl butyrate (pNPB), *p*-nitrophenyl laurate (pNPL), *p*-nitrophenyl caproate (pNPC), *p*-nitrophenyl caprylate (pNPCapr), *p*-nitrophenyl acetate (pNPA), and phenylboric acid were from Aldrich (Madrid, Spain). The remaining reagents were of analytical grade.

Extraction of Esterase from *T. claveryi* Ascocarps. Pieces of *T. claveryi* ascocarps, containing both peridium and gleba, were suspended in 0.1 M sodium phosphate buffer, pH 7.0, in a ratio 1:5 (w/v) before being homogenized with a mortar and pestle at 4 °C. The homogenate was then centrifuged at 10000*g* for 40 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 12% (w/v). The mixture was kept at 4 °C for 5 min and then warmed to 37 °C in a thermostatic bath. After ∼18 min, the solution became spontaneously turbid, due to 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 used as the enzyme source.

Protein Determination. The protein content was measured according to the bicinchoninic acid method (*23*) using BSA as the standard.

Determination of Phenolic Compounds. Phenolic compounds were measured spectrophotometrically using the Folin−Denis method in 80% ethanol using chlorogenic acid as the standard (*24*).

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

Substrate Specificity. The activity of *T. claveryi* esterase was assayed with different substrates: pNPA, pNPB, pNPC, pNPCapr, pNPL, and pNPP. Substrates were dissolved in ethanol or with TX-100. In the first case the reaction medium consisted of 940 µL of 50 mM phosphate buffer, pH 7.4, 10 µL of 10 mM substrate, and 0.0004 unit of enzyme. In the second set of experiments the substrates (4 mM) were emulsified with 40 mM TX-100 in 50 mM sodium phosphate buffer, pH 7.4. The reaction was started by adding 0.0004 unit of enzyme (final volume = 1 mL).

Enzyme Activity. Esterase activity was determined in a standard reaction medium consisting on 0.3 mM pNPB in 50 mM sodium phosphate buffer, pH 7.4 (final volume = 1 mL), recording *p*-nitrophenol production at 400 nm in a Uvikon 940 spectophotometer (Kontron). The extinction coefficient of *p*-nitrophenol at pH 7.4 was found to be 11825 M^{-1} cm^{-1}. The reaction was started by adding the enzyme. One unit of enzyme was defined as the amount of enzyme that produced 1 µmol of *p*-nitrophenol per minute at 25 °C in the standard reaction medium.

The experiments were performed in triplicate, and the mean and standard deviation (SD) were plotted.

Effect of pH. Because the absorbance of *p*-nitrophenol depends on the pH, the effect of pH on enzyme activity was calculated by incubating the enzyme in 10 mM sodium phosphate of different pH values (6.3, 6.7, 7.0, 7.4, and 7.7) and 0.3 mM pNPB. At different times, 1 mL aliquots were withdrawn from the reaction medium and placed in a cuvette with 1 mL of 0.1 M sodium phosphate buffer, pH 7.4. The

absorbance at 400 nm was measured and represented against time. Then, the Δabs per minute was calculated from this graph. A blank without enzyme was prepared for each pH following the same procedure.

Effect of Temperature. The optimum temperature was determined spectrophotometrically at 234 nm with an HP 8452A (Hewlett-Packard) diode array spectrophotometer using the standard reaction medium. The temperature of the buffer (between 22 and 70 °C) was adjusted by using an HP89090A Peltier accessory (Hewlett-Packard). Then, the substrate was added, and the reaction was started by adding the enzyme. A blank without enzyme was carried out at each temperature. The temperature stability was also studied in the standard reaction medium (pH 7.4). The enzyme was incubated at the desired temperature in a thermostatic bath. After 10 min, aliquots were withdrawn and the residual activity was measured at 25 °C.

Effect of Ionic Strength. The influence of the ionic strength on the initial rate was studied in a reaction medium containing sodium phosphate buffer, pH 7.4 (10, 25, 50, 50, and 100 mM). The substrate concentration was 0.3 mM and the amount of enzyme 0.0004 unit.

Inhibition with Phenylboric Acid. The reaction medium contained different concentrations of phenylboric acid in a reaction mixture containing 0.1 mM pNPB in sodium phosphate buffer, pH 7.4. In another experiment the initial rate was measured by fixing phenylboric acid concentrations (0, 0.5, or 0.8 mM) and changing the pNPB concentration. The reaction was started by adding the enzyme.

Histochemical Localization of Esterase in Ascocarps of *T. claveryi*. Sections of ascocarps (10 µm thick) were obtained using a Cryostat Reichert-Jung model 2700 Frigcut. The localization of esterase activity within the *Terfezia* ascocarp was investigated histochemically by incubating ascocarp sections for 45 min with 1 mM pNPB in 50 mM sodium phosphate buffer, pH 7.4. Controls were incubated for 5 min with 20 mM phenylboric acid and then with 1 mM pNPB for 45 min or with only buffer.

RESULTS AND DISCUSSION

Extraction of Esterase from *T. claveryi* Ascocarps. Different methods have been used to overcome the difficulties involved in enzyme purification from fungi (*25*). The use of TX-114 in the purification of proteins from plants and fungi presents many advantages (*26*), some of which are described below. It shows the special feature of forming clear solutions in buffers at 4 °C, whereas it separates into two phases at 25 °C due to the formation of large micellar aggregates (*27*). This characteristic has been used to separate integral proteins from hydrophilic proteins, because the former remain in the detergent-rich phase (*28*). TX-114 has been used in plant biochemistry in the removal of phenolic compounds from fruits or mushrooms (*25, 29*). Here, we use TX-114 to partially purify esterase from truffles. In addition to phenolic compounds, truffles present a high amount of lipids (*20*), which strongly interfere with the spectrophotometric characterization of esterase because they increase the turbidity of the reaction medium and may form micellar aggregates with certain hydrophobic substrates. With the method described in this paper, a reduction of 87% in the triglyceride content was obtained **(Table 1)**. In addition, 63% of phenols were removed with little protein loss during temperature-induced phase separation (62% of esterase recovery).

The fact that neither detergent nor sonication of the extract was needed in the extraction buffer and the partition of the enzyme in the aqueous phase suggests that this esterase was a soluble enzyme.

Substrate Specificity. Ascocarps of *T. claveryi* contain an enzymatic activity that is responsible for the hydrolysis of *p*-nitrophenyl esters. In principle, this reaction may be carried out by a lipase or by an esterase. According to Uchida (*30*), lipases can be distinguished from carboxyl esterases by their

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

step	vol (mL)	total activity (units)	total protein (mg)	specific activity (units/mg)	ourification (fold)	recovery (%)	total TAG[a] (mg)	total phenolic compd
crude extract	23	1.8	70	0.026	1.0	100	120	39.5
12% TX-114 supernatant	20	1.1	25	0.04	1.5	62	16	14.7

[a] TAG, triglycerides.
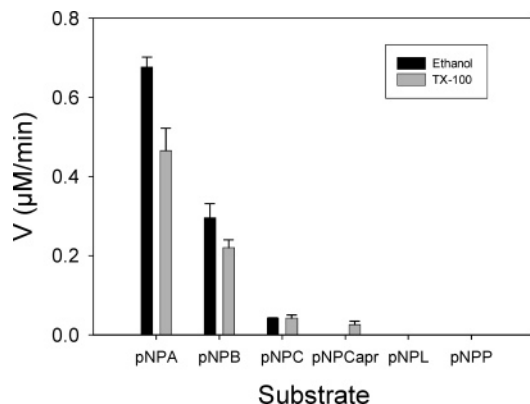
Figure 1. Substrate specificity of esterase from *T. claveryi*. Two different reaction media were assayed: black bars, substrates (final concentration = 100 μM) were dissolved in ethanol; gray bars, substrates (final concentration = 3.8 mM) were emulsified with TX-100. In both cases the reaction media contained 50 mM sodium phosphate buffer, pH 7.4, and 0.0004 unit of enzyme.

substrate spectra using pNPP (cleaved by lipase) versus pNPB (cleaved by esterases and sometimes also by lipases). To study the influence of fatty acid chain length on the hydrolytic rate and to distinguish between both enzymes, a series of *p*-nitrophenol esters were assayed. Two different reaction media reported in the literature were assayed. These assays differ in the procedure followed to prepare the substrate: emulsifying it with TX-100 (3.8 mM) or diluting it with ethanol (final concentration = 0.1 mM) (**Figure 1**). The results obtained in both assays were similar. Lipases differ from esterases in that their natural substrates are insoluble in water and their activity is maximal only when the enzyme is adsorbed to the oil/water interface. However, the results obtained with the two reaction media were similar, and no interfacial activation was observed, indicating that the enzyme extracted from *T. claveryi* was an esterase and not a lipase (*31*). These results were confirmed by the observation that the enzyme showed the maximum activity toward short-chain *p*-nitrophenyl esters such as pNPA and pNPB. Although the total substrate concentration was higher in the assay in which the substrate is solubilized with TX-100 (3.8 mM), the enzyme showed a higher activity when the substrates were dissolved in ethanol (0.1 mM). The activity on pNPC and pNPCapr was very low in both cases, and no reaction was observed when pNPL or pNPP were used as substrate.

These results suggest that the enzyme responsible for the hydrolysis of *p*-nitrophenyl esters was an esterase and not a lipase. In addition, when the activity of the extract toward the classical lipase substrate, olive oil, was assayed using the cupric acetate method described by Kwon and Rhee (*32*), the extract did not show lipolytic activity (data not shown).

Effect of Enzyme Concentration. The enzymatic character of the hydrolysis of pNPB was confirmed by measuring the initial reaction rate using different volumes of extract. The reaction medium contained 0.3 mM pNPB in 0.1M sodium phosphate buffer pH 7.4 and different volumes of enzyme. A
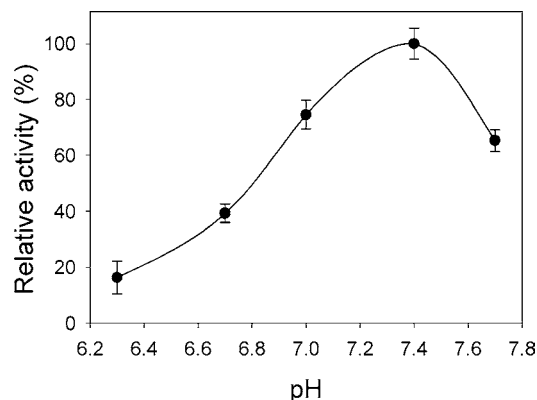

Figure 2. Effect of pH on esterase activity. The experiment was carried out as described under Materials and Methods.
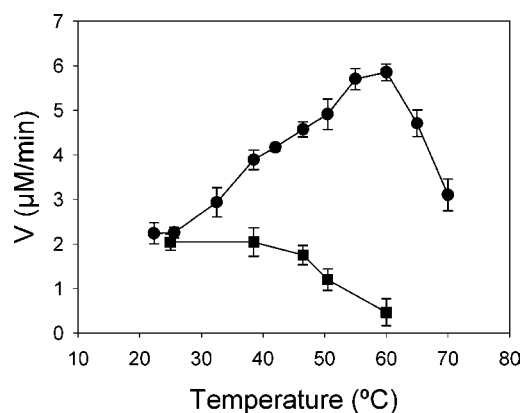

Figure 3. Determination of the optimum temperature of the esterase activity (●). The temperature of the buffer (50 mM sodium phosphate, pH 7.4) was adjusted using a Peltier accessory. The substrate (0.3 mM pNPB) was then added, and the reaction was started by adding the enzyme (0.002 unit). The temperature stability was also studied (■). The enzyme was incubated at the desired temperature in a thermostatic bath. After 10 min, aliquots were withdrawn and the residual activity was measured at 25 °C.

blank assay without enzyme was also carried out. The results obtained showed that the initial reaction rate was linear with the enzyme concentration (data not shown).

Effect of pH. The esterase assay was performed at different pH values (from 6.3 to 7.7) to determine the optimum pH for the enzyme toward pNPB (**Figure 2**). The activity of the enzyme steadily increased from pH 6.3 to 7.4 and then started to decrease. This pH optimum (pH 7.4) coincided with that described for esterase from *Sulfolobus solfataricus* (*33*) and was similar to that described for an esterase from *Aspergillus nomius* (*30*).

Effect of Temperature. The temperature profile for esterase (**Figure 3**) indicated a linear increase in activity from 25 to 55 °C. The optimum temperature for the enzyme activity was ~60 °C, above which the activity sharply decreased. However, the stability of the enzyme at 60 °C is low, and after 10 min of
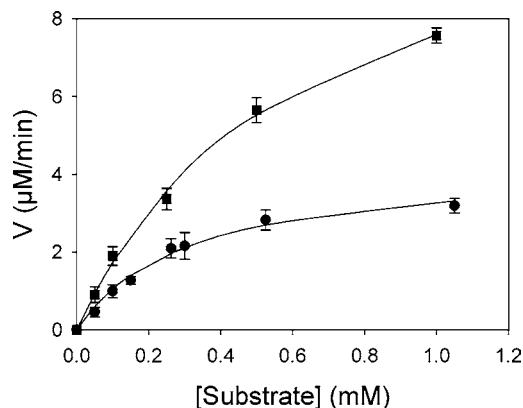
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Figure 4. Effect of substrate concentration on esterase activity. The reaction medium consisted of 0.002 unit of enzyme and different amounts of pNPA (■) or (●) pNPB in 50 mM sodium phosphate buffer, pH 7.4. Symbols represent the experimental points, and the solid line represents the fitting to the equation of Michaelis–Menten.
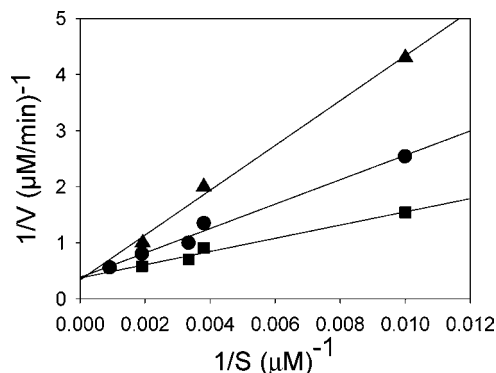

Figure 5. Effect of phenylboric acid. The assay was carried out by changing the pNPB concentration and keeping constant the inhibitor concentration: phenylboric acid, 0.8 mM (▲); phenylboric acid, 0.5 mM (●); control (■) and 0.0012 unit of enzyme in 0.1 M sodium phosphate buffer, pH 7.4.
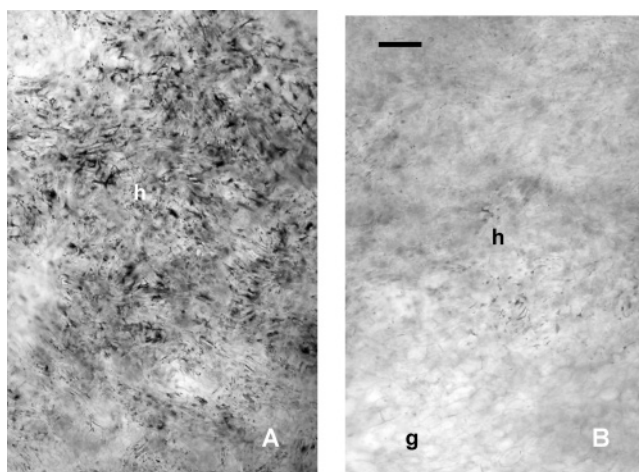

Figure 6. Localization of esterase activity in sections of *T. claveryi* ascocarps. The sections were incubated for 45 min with 1 mM pNPB in 50 mM sodium phosphate buffer, pH 7.4 (**A**). Controls were incubated for 5 min with 20 mM phenylboric acid and then with 1 mM pNPB (**B**): h, hypothecium; g, gleba. Bar = 40 μm.

incubation, the enzyme had lost 92% of the initial activity at this temperature. This low heat stability is similar to that reported for *Aspergillus nomius* esterase (*30*).

Kinetics Characterization of Esterase Activity. Whereas lipases need a minimum substrate concentration before they show high activity, esterases obey classical Michaelis–Menten kinetics (*31*), as was confirmed when the effect of substrate concentration on the activity was studied (**Figure 4**). The kinetic constants K_m and V_{max} toward pNPA and pNPB were calculated by nonlinear regression fitting (*34*) of the experimental points to the equation of Michaelis–Menten. This fitting is represented by the solid line between the experimental points. The values obtained for K_m at pH 7.4 were 0.3 mM for pNPB and 0.6 mM for pNPA, the latter value being similar to that obtained by Uchida et al. (*30*) for *Aspergillus nomius* esterase. The V_{max} values for pNPB and pNPA were 68.7 and 195 μM/min/mg of protein, respectively. The catalytic efficiency (V_{max}/K_m), which expresses the initial velocity at substrate concentrations below the K_m, were more similar: 0.23 for pNPB and 0.32 for pNPA.

Because the enzyme presents a higher affinity for pNPB and this substrate is more stable than pNPA, the rest of the assays were carried out using pNPB.

Effect of Ionic Strength. The ionic strength did not affect the initial rate, and the rest of the assays were carried out with 50 mM phosphate buffer (data not shown).

Effect of Phenylboric Acid. Most lipases and esterases contain the consensus sequence motif Gly-x-Ser-x-Gly (where x represents an arbitrary amino acid residue) around the active site serine (*31*). Phenylboric acid is a reversible inhibitor of most serine esterases (*35*), and it binds at or near the active site serine (*36*). This compound also inhibited the esterase from *T. claveryi*, which indicates that serine residues were involved in the enzyme activity. The nature of this inhibition was studied by changing the substrate concentration at different concentrations of phenylboric acid (**Figure 5**). The 1/*V* versus 1/[S] plots were linear, and all intersected at the 1/*V* axis, indicating that phenylboric acid is a competitive inhibitor of *T. claveryi* esterase. The K_i obtained for this compound was 294 μM.

Localization of Esterase Activity. Localization of fungal enzymes at the whole tissue level using histochemical methods is a useful tool in the characterization and elucidation of the function of many enzymes (*37, 38*). However, the application of this method has been hampered by endogenous substrates, inhibitors, and phenolics that can inactivate enzymes (*39*). A

reliable method for locating enzymes in whole tissue sections would have considerable application to fungal biochemistry. Esterase has been localized in hyphae of pathogenous fungi (*11, 14*). To the best of our knowledge, this is the first report on the localization of this activity within the ascocarp of an edible fungi (**Figure 6**). Unlike *Agaricus bisporus* mushrooms (*40*), the color of *T. claveryi* ascocarps permitted the staining for enzymatic activities without blotting the tissue sections onto nitrocellulose (*38*). Esterase was localized only in the hypothecium (**Figure 6A**), and it was absent in the peridium and gleba (data not shown). When sections were incubated with phenylboric acid prior to the addition of pNPB, no reaction was observed (**Figure 6B**). Because the localization of esterase activity in a mushroom is reported for the first time, the results presented cannot be compared with any other.

In conclusion, this paper reports for the first time the presence of an esterase activity in *T. claveryi* ascocarps. The results obtained suggest that the enzyme responsible is an esterase and not a lipase. It is a soluble enzyme that displays its maximum activity at pH 7.4 and 60 °C. Inhibition by phenylboric acid suggests the presence of serine residues in its active center. Esterase activity was detected only in the hypothecium.

ABBREVIATIONS USED

ACKNOWLEDGMENT

LITERATURE CITED

(1) Chahinian, H.; Nini, L.; Boitard, E.; Dubes, J. P.; Comeau, L. C.; Sarda, L. Distinction between esterases and lipases: a kinetic study with vinyl esters and TAG. *Lipids* **2002** *37*, 653−662.

(2) Fries, L. L. M.; Pacovsky, R. S.; Safir, G. R. Expression of isoenzymes altered by both *Glomus intraradices* colonization and formononetin application in corn (*Zea mays* L.) roots. *Soil Biol. Biochem.* **1996**, *28*, 981−988.

(3) Timonen, S.; Sen, R. Heterogeneity of fungal and plant enzyme expression in intact Scots pine *Suillus bovinus* and *Paxillus involutus* mycorrhizospheres developed in natural forest humus. *New Phytol.* **1998**, *138*, 355−366.

(4) Vázquez, M. M.; César, S.; Azcón, R.; Barea, J. M. Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum*, *Pseudomonas*, *Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. *Appl. Soil Ecol.* **2000**, *15*, 261−272.

(5) Hepper, C. M.; Sen, R.; Maskall, C. S. Identification of vesicular-arbuscular mycorrhizal fungi in roots of leek (*Allium porrum* L.) and maize (*Zea mays* L.) on the basis of enzyme mobility during polyacrylamide gel electrophoresis. *New Phytol.* **1986**, *102*, 529−539.

(6) Hepper, C. M.; Sen, R.; Azcon-Aguilar, C.; Grace, C. Variation in certain isozymes amongst different geographical isolates of the vesicular-arbuscular mycorrhizal fungi *Glomus clarum*, *Glomus monosporum* and *Glomus mosseae*. *Soil Biol. Biochem.* **1988**, *20*, 51−59.

(7) Yokono, T.; Tamai, Y.; Azuma, T.; Sakuma, Y.; Miura, K.; Kojima, Y.; Sunagawa M.; Ohmasa, M. Karyotype analysis of interspecific fusants of basidiomycetes by pulsed-field gel electrophoresis. *J. Wood Sci.* **2000**, *46*, 480−484.

(8) Bonefant Magne, M.; Magne, C.; Esnault, M. A.; Lemoine, C. Characterization of cultivated strains of a new edible mushroom: *Stropharia rugoso-annulata*. 1. Protein variability. *Cryptogam. Mycol.* **1997**, *18* (3), 255−265.

(9) Matsumoto, T.; Mimura, K.; Fukumasa Nakai, Y. Isozyme variation and genetic relatedness among natural populations of *Pleurotus ostreatus*. *J. Gen. Appl. Microbiol.* **1995**, *41*, 487−497.

(10) Sugui, J. A.; Pascholati, S. F.; Kunoh, H.; Howard, R. J.; Nicholson, R. L. Association of *Pestalotia malicola* with the plant cuticle: visualization of the pathogen and detection of cutinase and non-specific esterase. *Physiol. Mol. Plant Pathol.* **1998**, *52*, 213−221.

(11) Hsieh, T. F.; Huang, J. W.; Hsiang, T. Light and scanning electron microscopy studies on the infection of oriental lily leaves by *Botrytis elliptica*. *Eur. J. Plant Pathol.* **2001** *107*, 571−581.

(12) Deising, H.; Nicholson, R. L.; Haug, H.; Howard, R. J.; Mendgen, K. Adhesion pad formation and the involvement of cutinase and esterases in the attachment of uredospores to the host cuticle. *Plant Cell* **1992**, *4*, 1101−1111.

(13) Pascholati, S. F.; Deising, H.; Leite, B.; Anderson, D.; Nicholson, R. L. Cutinase and non-specific esterase activities in the conidial mucilage of *Colletotrichum graminicola*. *Physiol. Mol. Plant Pathol.* **1993**, *42*, 37−51.

(14) Jansson, H. B.; Åkesson, H. Extracellular matrix, esterase and the phytotoxin prehelminthosporol in infection of barley leaves by *Bipolaris sorokiniana*. *Eur. J. Plant Pathol.* **2003**, *109*, 599−605.

(15) Cristiani, G.; Monnet, V. Food micro-organisms and aromatic ester synthesis. *Sci. Aliments* **2001**, *21*, 211−230.

(16) Nardi, M.; Fiez-Vandal, C.; Tailliez, P.; Monnet, V. The EstA esterase is responsible for the main capacity of *Lactococcus lactis* to synthesize short chain fatty acid esters *in vitro*. *J. Appl. Microbiol.* **2002**, *93*, 994−1002.

(17) McSweeney, P. L. H.; Sousa, M. J. Biochemical pathways for the production of flavour compounds in cheeses during ripening: a review. *Lait* **2000**, *80*, 293−324.

(18) Horsted, M. W.; Dey, E. S.; Holmberg, S.; Kielland-Brandt, M. C. A novel esterase from *Saccharomyces carlsbergensis*, a possible function for the yeast *TIP1* gene. *Yeast* **1998**, *14*, 793−803.

(19) Murcia, M. A.; Martínez-Tomé, M.; Jiménez, A. M.; Vera, A. M.; Honrubia, M.; Parras, P. Antioxidant activity of edible fungi (truffles and mushrooms): losses during industrial processing. *J. Food Prot.* **2002**, *65*, 1614−1622.

(20) Murcia, M. A.; Martínez-Tomé, M.; Vera, A.; Morte, A.; Gutiérrez, A.; Honrubia, M.; Jiménez, A. M. Effect of industrial processing on desert truffles *Terfezia claveryi* Chatin and *Picoa juniperi* Vittadini: proximate composition and fatty acids. *J. Sci. Food Agric.* **2003**, *83*, 535−541.

(21) Morte, A.; Honrubia, M. Improvement of mycorrhizal synthesis between micropropagated *Helianthemum almeriense* plantlets with *Terfezia claveryi* (desert truffle). In *Science and Cultivation of Edible Fungi*; Elliott, T. J., Ed.; Balkema: Rotterdam, The Netherlands, 1995; Vol. 2, pp 863−868.

(22) Morte, A.; Lovisolo, C.; Schubert, A. Effect of drought stress on growth and water relations of the mycorrhizal association *Helianthemum almeriense−Terfezia claveryi*. *Mycorrhiza* **2000**, *10*, 115−119.

(23) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76−85.

(24) Kidron, M.; Harel, M.; Mayer, A. M. Catechol oxidase activity in grapes and wine. *Am. J. Enol. Vitic.* **1978**, *219*, 30−35.

(25) Núñez-Delicado, E.; Bru, R.; Sánchez-Ferrer, A.; García-Carmona, F. TX-114 aided purification of latent tyrosinase. *J. Chromatogr. B* **1996**, *680*, 105−112.

(26) Sánchez-Ferrer, A.; Pérez-Gilabert, M.; Núñez, E.; Bru, R.; García-Carmona, F. Triton X-114 phase partitioning in plant protein purification. *J. Chromatogr. A* **1994**, *668*, 75−83.

(27) Bordier, C. Phase separation of integral membrane proteins in TX-114 solution. *J. Biol. Chem.* **1981**, *256*, 1604−1607.

(28) Pryde, J. G.; Philips, J. H. Fractionation of membrane proteins by temperature-induced phase separation in TX-114 application to subcellular fractions of the adrenal medulla. *Biochem. J.* **1986**, *233*, 525−533.

(29) Pérez-Gilabert, M.; García-Carmona, F. Characterization of catecholase and cresolase activities of eggplant polyphenol oxidase. *J. Agric. Food Chem.* **2000**, *48*, 695−700.

(30) Uchida, H.; Kurakata, Y.; Sawamura, H.; Inamura, N.; Kotani, T.; Uwajima, T. Purification and properties of an esterase from *Aspergillus nomius* HS-1 degrading ethylene glycol dibenzoate. *FEMS Microbiol. Lett.* **2003**, *223*, 123−127.

(31) Bornscheuer, U. Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiol. Rev.* **2002**, *26*, 73−81.

(32) Kwon, D. Y.; Rhee, J. S. A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. *J. Am. Oil Chem. Soc.* **1986**, *63*, 89−92.

(33) Kim, S.; Lee, S. B. Thermostable esterase from a thermoacidophilic archaeon: purification and characterization for enzymatic resolution of a chiral compound. *Biosci., Biotechnol., Biochem.* **2004**, *68*, 2289−2298.

(34) Marquardt, D. W. An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Ind. Appl. Math.* **1963**, *11*, 431–441.

(35) Gjellesvik, D. R.; Lombardo, D.; Walter, B. T. Pancreatic bile salt dependent lipase from cod (*Gadus morhua*): purification and properties. *Biochim. Biophys. Acta* **1992**, *1124*, 123–124.

(36) Garner, C. W. Boronic acid inhibitors of porcine pancreatic lipase. *J. Biol. Chem.* **1980**, *255*, 5064–5068.

(37) Miranda, M.; Zarivi, O.; Bonfigli, A.; Amicarelli, F.; Aimola, P.; Ragnelli, A. M.; Pacioni, G. Melanogenesis tyrosinase expresión, and reproductive differentiation in black and white truffles (ascomycotina). *Pigm. Cell Res*. **1997**, *10*, 46–53.

(38) Pérez-Gilabert, M.; Morte, A.; Honrubia, M.; García-Carmona, F. Partial purification, characterization, and histochemical localization of fully latent desert truffle (*Terfezia claveryi* Chatin) polyphenol oxidase. *J. Agric. Food Chem.* **2001**, *49*, 1922–1927.

(39) Spruce, J.; Mayer, A. M.; Osborne, D. J. A simple histochemical method for locating enzymes in plant tissues using nitrocellulose blotting. *Phytochemistry* **1987**, *26*, 2901–2903.

(40) Moore, B. M.; Kang, B.; Flurkey, W. H. Histochemical localization of mushroom tyrosinase in whole tissue sections on nitrocellulose. *Histochemistry* **1989**, *90*, 379–381.