Partial Purification, Characterization, and Histochemical Localization of Fully Latent Desert Truffle (*Terfezia Claveryi* Chatin) Polyphenol Oxidase

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In the present paper, a fully latent polyphenol oxidase (PPO) from desert truffle *(Terfezia claveryi* Chatin) ascocarps is described for the first time. The enzyme was partially purified by using phase partitioning in Triton X-114 (TX-114). The achieved purification was 2-fold from a crude extract, with a 66% recovery of activity. The interfering lipids were reduced to 13% of the original content. In addition, the purification gave rise to a reduction of phenolic compounds to only 37.5%, thus avoiding the postpurification tanning of the enzyme. Latent PPO was activated by the anionic surfactant sodium dodecyl sulfate (SDS) or by incubation with trypsin. The amount of SDS necessary to obtain a maximum activation was dependent on the nature of the substrate. The use of SDS also permitted the histochemical localization of the latent enzyme within the ascocarp. *Terfezia* polyphenol oxidase was kinetically characterized using two phenolic substrates (L-DOPA and *tert*-butylcatechol). The latter substrate presented inhibition at high substrate concentration with a K_{si} of 6.3 mM. Different inhibiting agents (kojic and cinnamic acid, mimosine and tropolone) were also studied, tropolone being the most effective.

Keywords: *Ascocarp; desert truffle; mycorrhizal hypogeous fungi; SDS; Triton X-114*

INTRODUCTION

Desert truffles are a complex concept of mycorrhizal hypogeous fungi including several species of the genera *Balsamia*, *Picoa*, *Terfezia*, *Tirmania*, and *Tuber*, whose distribution is limited to semi-arid and arid conditions (*1*). The most important desert truffles species are those included in the genera *Terfezia* and *Balsamia* because of their highly appreciated edible and commercial value, particularly in Mediterranean and Arabic peninsula countries. Mainly morphological (*1, 2*) and bromatological (*3−6*) studies have been carried out with desert truffles; virtually no biochemical investigations about their metabolism have been performed.

Tyrosinase or polyphenoloxidase (PPO) (monophenol, dihydroxyphenilalanine:oxygen oxidoreductase, EC 1.14.18.1) is a bifunctional, copper-containing enzyme widely distributed on the phylogenetic scale. This enzyme uses molecular oxygen to catalyze the oxidation of monophenols, for example, tyrosine, to their corresponding *o*-diphenols (monophenolase-*o*-cresolase activity) and their subsequent oxidation to *o*-quinones (diphenolase or catecholase activity). For a review on both activities see ref 7 . The *o*-quinones thus generated polymerize to form melanin through a series of subsequent enzymic and nonenzymic reactions. Although the physiological function of tyrosinase in fungi is not yet understood, melanin synthesis is correlated with differentiation of reproductive organs and spore formation, virulence of pathogenic fungi, and tissue protection after injury (*8−13*). In addition, polyphenol oxidase is responsible for the undesired enzymatic browning of mushrooms that takes place during senescence or damage during post harvest handling.

The isolation of enzymes in fungi is complicated by the existence of a large variety of compounds that can interact with enzymes and change their characteristics (*14*). Most of the methods developed to overcome this problem lead to partial enzyme inhibition or denaturalization, or the uncontrolled activation of the native inactive forms (latent enzymes). The special characteristics of polyoxyethylene-type nonionic detergents, especially Triton X-114 (TX-114), permit the removal of interfering substances during fungi protein purification and the extraction of enzymes in their native form without being activated (*15, 16*).

In this paper, the presence of polyphenol oxidase in *Terfezia claveryi* is reported for the first time. The enzyme has been purified in a fully latent state using phase partitioning in TX-114, histochemically localized within the ascocarp at two maturity stages and kinetically characterized.

MATERIALS AND METHODS

Fungal Material. Young (without asci) and mature (with asci and spores) ascocarps of *T. claveryi* Chatin were collected on an Alep pine plantation, associated with *Helianthemum almeriense* Pau shrubs, in Zarzadilla de Totana (Lorca, Murcia, Spain) and used a few hours after collection or after storage at −20 °C.

Reagents. Bicinchoninic acid, bovine serum albumin (BSA), chlorogenic acid, cinnamic acid, copper sulfate pentahydrate 4%, L-dihydroxyphenylalanine (L-DOPA), L-mimosine, Folin

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Table 1. Partial Purification of *Terfezia* Polyphenol Oxidase

	vol (ml)	total protein (mg)	specific activity (U/mg)	purification (fold)	recovery (%)	total TAG (mg)	total phenolic compounds (mg)
crude extract	4.1	36.1	2	1.0	100	3.8	6.4
supernatant 12% TX-114	3.6	12.1	4	2	66	0.5	2.4

reagent, "Infinity" triglycerides reagent kit, kojic acid, mimosine, sodium dodecyl sulfate (SDS), and tropolone were purchased from Sigma (Madrid, Spain). *tert*-Butylcatechol and TX-114 were obtained from Fluka (Madrid, Spain). The remaining reagents were of analytical grade.

Enzyme Purification. Pieces of *Terfezia* ascocarps, containing both peridium and gleba, after suspension in 0.1 M sodium phosphate buffer pH 7.0 in a ratio 1:5 (w/v) were homogenized with a mortar and pestle at 4 °C. The homogenate was then centrifuged at 15000*g* for 20 min. 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%. The mixture was kept at 4 °C for 10 min and then warmed to 37 °C in a thermostatic bath. After 30 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 enzymatic extract. The enzyme remained stable at -20 °C for > 3 months.

Enzyme Activity. Catecholase activity was determined spectrophotometrically using L-DOPA and *tert*-butylcatechol (TBC), recording dopachrome production at 475 nm (ϵ = 3600 M^{-1} cm^{-1}) and *tert*-butylquinone production at 400 nm (ϵ = 1682 M^{-1} cm^{-1}).

Experiments were performed in triplicate and the mean and standard deviation (SD) were plotted. One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of *tert*-butylquinone/min in a reaction medium containing 2 mM SDS, and 5 mM TBC in 0.1 M phosphate buffer, pH 7.0.

Protein Determination. The protein content was measured according to the method of bicinchoninic acid (*17*) using BSA as the standard.

Determination of Phenolic Compounds. Phenolic compounds were measured spectrophotometrically according to the Folin-Denis method in 80% ethanol using chlorogenic acid as the standard (*18*).

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

Electrophoresis. Nondenaturing polyacrylamide gel electrophoresis (PAGE) was carried out at 4 °C on 7.5% slabs in a Mini Protein cell (Bio-Rad). After the electrophoresis run, the gel was incubated for 15 min in 0.1 M phosphate buffer (pH 5.5) and then with 10 mM L-DOPA in the same buffer.

Histochemistry for Catecholase Activity. 10-μm-thick sections of both young and mature ascocarps were obtained using a Cryostat Reichert-Jung model 2700 Frigcut.

The localization of latent catecholase activity within the *Terfezia* ascocarp was investigated histochemically by incubating ascocarp sections for 2 h with 10 mM L-DOPA containing 2.5 mM SDS in 0.1 M phosphate buffer (pH 5.5). Controls were incubated with L-DOPA without SDS, with 50 μM tropolone, or with only buffer.

RESULTS AND DISCUSSION

Purification. Enzyme purification in fungi extracts becomes difficult because of the presence of a large variety and quantity of secondary products that can bind tightly to the enzymes and change their characteristics (*14*). To overcome this, different methods have been developed such as acetone powders, ammonium-sulfate fractionation, salts, insoluble polymers, and detergents. Among the last, TX-114 shows the special feature of forming clear solutions in buffers at 4 °C, while it separates into two phases at 25 °C due to the formation of large micellar aggregates (*19*). This characteristic has been used to separate integral proteins from hydrophilic proteins, because the former remain in the detergent-rich phase (*20*). Recently, TX-114 has found another use in plant biochemistry in the removal of phenolic compounds from fruits or mushrooms (*14, 21, 22*).

Figure 1. Nondenaturing electrophoresis (7.5% gel) of desert truffle PPO. The slab was stained with 10 mM L-DOPA plus 2.5 mM SDS in 0.1 M sodium phosphate buffer (pH 5.5). The lane contained 44 μg of partially purified PPO.

Truffles, in addition to phenolic compounds, present a high amount of lipids (*4, 5*) that strongly interfere with the spectrophotometric characterization of PPO, 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 triglycerides content was obtained (Table 1). In addition, 62.5% of phenols were removed with little protein loss during temperature-induced phase separation (66% of PPO recovery) (Table 1). The degree of purification and recovery cannot be compared to any published data on truffles' PPO, but it was similar to that found when using TX-114 to purify *Agaricus bisporus* PPO (*14*). The reduction of the phenolic content by TX-114 was sufficient to avoid browning of the enzyme solution even after months of storage at −20 °C.

The partially purified *Terfezia* PPO appeared as a single activity band in PAGE when L-DOPA was used as substrate (Figure 1). This is in contrast to the multiplicity found in PAGE for PPO of different truffles of the genus *Tuber* (*23*) and for other soluble plant PPO, which may be due to covalent reactions of the enzyme with its generated *o*-quinones (*24*).

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 PPO was a soluble enzyme, as is *A. bisporus* (*25*) and *Neurospora crassa* PPO (*26*).

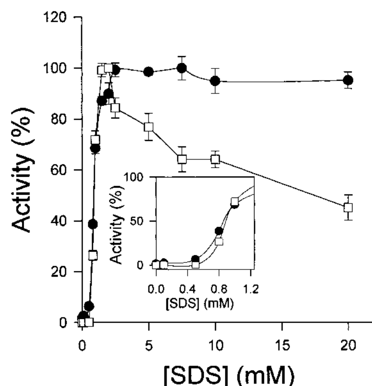

Figure 2. Percentage of activation of *Terfezia* PPO with different concentrations of SDS. The reaction medium contained 0.07 unit PPO plus 2.5 mM TBC in 0.1M phosphate buffer pH 7.0 (□) or 0.5 mM L-DOPA in 0.1M phosphate buffer pH 5.5 (●). The inset shows the activity profile at lower SDS concentrations.

Activation of PPO. One unusual and intriguing characteristic of PPO is its ability to exist in an inactive or latent state. This latency has been described especially in plants, where it is very common in thylakoid-bound polyphenoloxidases (*27, 28*). The PPO from *A. bisporus* has been found in both active and latent forms (*25*), and sample preparation apparently has a strong influence on extractability and stability of the latent enzyme. PPO can be released from latency or activated by a variety of treatments or agents (29), including acid and base shock; urea; anionic detergents, such as SDS; proteases; and fatty acids. Use of SDS as an activating agent is particularly interesting because few enzymes are known to be activated by SDS, in contrast to many enzymes which are inactivated by this compound.

The enzyme obtained by the reported extraction method was fully latent, both in mature and inmature ascocarps. To detect any catecholase activity, the presence of SDS or trypsin (data not shown) in the reaction medium was necessary. The effect of SDS depended on the substrate used. In Figure 2, the results obtained with different concentrations of SDS using TBC or L-DOPA as substrates are shown. In both cases, the partially purified PPO was activated in a sigmoidal manner (Figure 2 inset), with increasing SDS concentrations below the CMC for the detergent, 3.5 mM at pH 6.0 (*29*). For both substrates, little activation occurred until 0.5 mM SDS. The maximum activation for TBC was obtained using 2 mM SDS. A further increase in SDS concentration gave rise to a decrease in the activity. A different trend was obtained for L-DOPA, where an increase in SDS from 2.5 to 20 mM produced only a 5% decrease in activity. The mechanism of activation as well as physical changes accompanying SDS have yet to be elucidated, and *Terfezia* PPO, one of the few PPO that can be easily purified in a fully latent state, might be a useful tool in such research.

Effect of pH. The pH profiles of catecholase activity were dependent on the substrate used. In the case of L-DOPA, the enzyme was active between pH 3.5 and pH 7.0, showing a maximum between pH 5.5 and 6.0 (Figure 3 open symbols). A considerable increase in activity at pH 5.0 was observed when acetate buffer was replaced by phosphate buffer. These results differ from those reported by Miranda et al. (*23*) for PPO from *Tuber brumale* whose activity toward L-DOPA is essentially independent of pH in the range 5−7. When

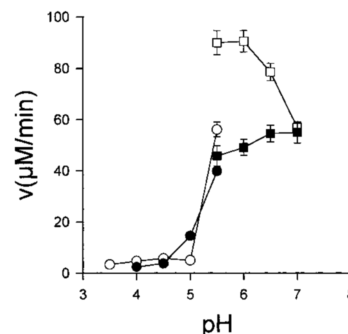

Figure 3. Effect of pH on the catecholase activity of *Terfezia* PPO. The reaction medium consisted of 2 mM SDS, 2.5 mM TBC (solid symbols), or 2.5 mM SDS and 9.6 mM L-DOPA (open symbols), 0.07 unit PPO, and 0.1M acetate (pH 4.0−5.5, circles) or phosphate (pH 5.5−7.0, squares).

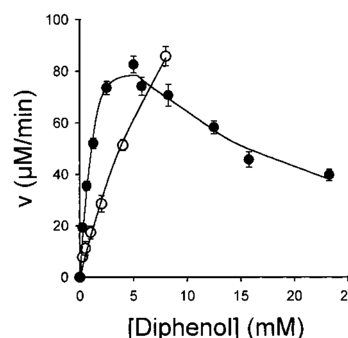

Figure 4. Effect of substrate concentration on catecholase activity of PPO. The reaction medium consisted of 2.5 mM SDS, 0.07 unit of PPO, and different amounts of L-DOPA in 0.1 M phosphate buffer, pH 5.5 (○) or different concentrations of TBC in 0.1M phosphate buffer pH 7.0 (●).

TBC was used as substrate, the pH profile obtained was slightly different (Figure 3, solid symbols). In this case, the enzyme showed a broad maximum at pH ∼ 7.0. Higher pHs were not assayed to avoid interferences produced by substrates autoxidation.

Effect of Substrate Concentration.The kinetic parameters (V_{max} and K_m) of L-DOPA and TBC oxidation were studied at the optimum pH of each substrate (Figure 4). In the case of L-DOPA, the initial rate showed a hyperbolic dependence with respect to substrate concentration; the low solubility of L-DOPA made the saturation of PPO impossible (Figure 4). K_m and V_{max} were calculated by nonlinear regression fitting (*30*) of the experimental points to the following equation

$$V = \frac{V_{max}[substrate]}{K_m + [substrate]} \quad (1)$$

This fitting is represented by the solid line between the experimental points. The values obtained for V_{max} and K_m gave values of 7.1 μmol min^{-1}(mg of protein)$^{-1}$ and 12.7 mM, respectively. This K_m is ∼30-fold higher than the one reported for *T. brumale* PPO (*23*). However, these results are not comparative, because the activation state of both enzymes is different, and the presence of SDS in the reaction medium can influence the value of K_m (*28*). The oxidation of TBC displayed a decrease in enzymatic activity when the substrate concentration was raised and showed a typical kinetic profile of substrate inhibition. These results were kinetically analyzed by nonlinear regression fitting (*30*) of the

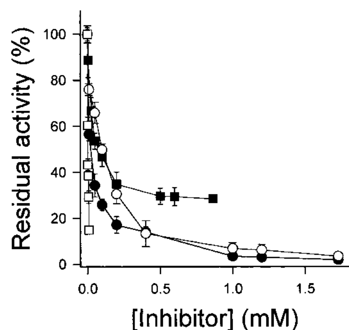

Figure 5. Effect of different inhibitors on catecholase activity of PPO using L-DOPA as substrate. The reaction medium consisted of 2.5 mM SDS, 0.07 unit of PPO, 1 mM L-DOPA, and different cocentrations of the following inhibitors: (□) tropolone; (■) cinnamic acid; (●) kojic acid or (○) L-mimosine in 0.1 M phosphate buffer, pH 5.5.

experimental points to the following equation

$$V = \frac{V_{\max}[TBC]}{K_m + [TBC] + \frac{[TBC]^2}{K_{si}}} \quad (2)$$

where K_{si} is the substrate inhibition constant. The kinetic parameters obtained with this substrate were V_{max}, 5.9 μmol min^{-1}(mg of protein)$^{-1}$; K_m, 2.64 mM; and K_{si}, 6.35 mM. The inhibition observed cannot be attributed to an effect of SDS on TBC because the same profile was obtained using different concentrations of SDS (data not shown).

Although SDS is known to affect the pH optimum and the kinetic constants V_{max} and K_m of PPO (*28*), these parameters could not be determined in the absence of SDS, because under these conditions, the enzyme was completely inactive.

Effect of Different Inhibitors. The effect of specific inhibitors (kojic and cinnamic acid, mimosine and tropolone) on the catecholase activity of *Terfezia* PPO using L-DOPA as substrate was also analyzed. This study is particularly interesting because the inhibition of PPO activity might have an influence on the mycelium growth, as reported by Poma et al. (*31*) for the white truffle *Tuber borchii*. Figure 5 shows the percentage of residual activity obtained with different concentrations of these compounds. Among these, the substrate analogue tropolone was the most effective inhibitor. This compound of the 2-hydroxy-1-one family completely inhibited the enzyme at 15 μM, which is in agreement with the values reported by Núñez-Delicado et al. (*14*) for mushroom PPO. Kojic acid, a fungal metabolite produced by many species of *Aspergilus* an*d Penicillium* (*32, 33*) was also a potent inhibitor of *Terfezia* PPO, and only 2% of the residual activity could be detected when a concentration of 1.5 mM was used. The profile of inhibition obtained using mimosine was similar to that of kojic acid, although at lower concentrations, kojic acid was a more effective inhibitor. Cinnamic acid had relatively little inhibiting effect on this enzyme. However, because it has been reported that the inhibition by this compound is pH-dependent (*34*), its effect at different pHs should be studied.

Histochemical Localization. In Figure 6A,B, the localization of PPO activity within mature ascocarps is presented. This activity is localized in the peridium, hypotecium, asci, spores, and ascogenic hyphae of the mature ascocarps. Essentially, the same localizations

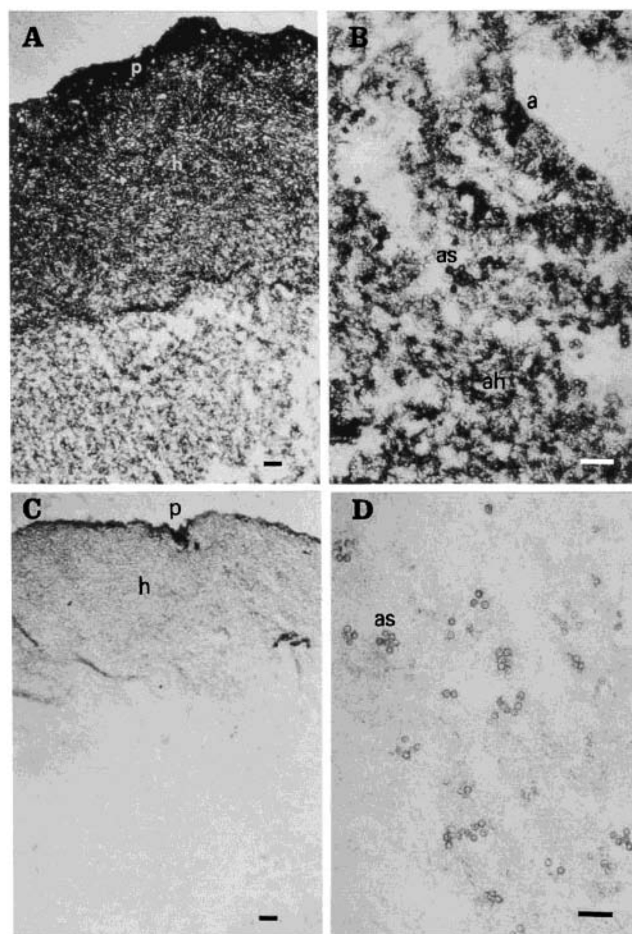

Figure 6. Localization of PPO activity in sections of *Terfezia claveryi* mature ascocarps. The sections were incubated for 2 h in 10 mM L-DOPA containing 2.5 mM SDS in 0.1 M phosphate buffer pH 5.5 (A and B) or in 0.1 M phosphate buffer pH 5.5 (C and D). p, peridium; h, hypothecium; a, asci; as, ascospores; ah, ascogenic hyphae. Bars = 40 μm.

Table 2. Variation of Ascocarp Chemical Composition

maturity of ascocarp	TAG[a]	protein[a]	phenols[a]	PPO[b]
young	18.1 ± 0.5	173 ± 3	30.6 ± 2	178 ± 17
mature	33.3 ± 1.4	250 ± 19	27.6 ± 5.4	660 ± 65

[a] mg/g dry weight. [b] Enzyme units.

were observed within the young ascocarps (data not shown). At this stage, no PPO activity could be detected in asci and spores, because they were not yet formed in the unripe ascocarps used in this experiment. In control sections of both mature (Figure 6C,D) and young ascocarps, no reaction was observed. These results agree well with those obtained with *Tuber* species (*13*) being the catecholase localization in *T. claveryi,* more similar to the white truffles *Tuber magnatum*, *Tuber puberulum* and *Tuber spaerospermum*, which do not show evident ramification of the basal cavity, than to *Tuber melanosporum* and *Tuber excavatum*, which possess a different ascocarp morphology. The presence of PPO activity in mature ascocarps of *T. claveryi* at even higher levels than the ones detected in young ascocarps (Table 2) contrasts with the results reported by Ragnelli et al. (*35*) in sub-adult ascocarps of *T. melanosporum* and *Tuber aestivum,* where no PPO activity was observed, even though a feeble Schmorl's reaction for melanin occurred. These discrepancies, in addition to the different genus of desert truffles, can be attributed to the

different state of activation of PPO that was not analyzed by these authors. In the present study, the presence of SDS in the incubation medium makes possible the histochemical localization of a fully latent enzyme that otherwise would be described as not present in this fungus.

Variation of Ascocarps' Chemical Composition. Table 2 shows the value of some constituents of *Terfezia* ascocarps at two stages of maturity. The level of phenolic compounds was considerable lower than that reported by Nuñez-Delicado et al. (*14*) for *A. bisporus*, and no significant change with the developmental stage has been observed. The contents of proteins and lipids are comparable to those reported by Sawaya et al. (*5*) for different forms of *T. claveryi* and confirm the nutritional quality of this tuber (*6, 36*). An increase in both lipid and protein contents was observed. Similar results were obtained by Ewaze et al. (*37*) with *Picoa juniperi vitt.* ascocarps. This increase in protein content found at the mature stage of development may reflect the high metabolic demands made for the maturation of asci and ascospores within the fruit body. On the other hand, a remarkable increase in PPO activity was observed. Although the protein content was 1.4-fold higher in the mature stage than in the immature stage, PPO activity was 3.7-fold higher. Thus, the elevation in PPO activity cannot be simply attributed to the general increment in the level of proteins. This result, together with the localization of PPO in spores, suggests that PPO could play an important role in the maturation of asci and ascospores.

CONCLUSIONS

TX-114 has been extensively used to purify plant enzymes since it was first used by our group in 1989 (*21, 38, 39*). This nonionic detergent avoids the use of drastic methods, such as acetate powder of insoluble polymers, which inhibit or activate latent enzymes (for a review, see refs *15, 16*). In this paper, the use of TX-114 for the elimination of interfering lipids in an extract of the mycorrhizic fungus *T. claveryi* is reported. With this method, one of the few fully latent PPOs has been extracted and characterized.

ABBREVIATIONS USED

CMC, critical micellar concentration; L-DOPA, L-dihydroxyphenylalanine; TX-114, Triton X-114; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; TBC, *tert*-butylcatechol.

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