Purification and Characterization of Two Enzymes Involved in the Quality of Desert Truffles: Tyrosinase and Lipoxygenase

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Abstract

This contribution presents a review of the purification and characterization of two enzymes involved in the quality of desert truffles: lipoxygenase and tyrosinase. Both enzymes were extracted using phase partitioning in Triton X-114, a method which permits the elimination of most of the lipids and phenols and to obtain a discrete degree of purification.

Tyrosinase from \textit{T. claveryi} is a fully latent enzyme which was activated by the anionic surfactant SDS. The use of SDS also permitted the histochemical localization of the latent enzyme within the ascocarp. Tyrosinase was kinetically characterized using L-DOPA as substrate. The effect of different inhibiting agents was also studied, tropolone being the most effective.

The results obtained indicate that lipoxygenase from \textit{T. claveryi} ascocarps is a soluble enzyme with a molecular weight of 66 kDa, which displays its maximum activity at pH 7.0 using linoleic acid as substrate. When using this fatty acid, the product of this reaction was the corresponding 13-hydroperoxide.

1 INTRODUCTION

Desert truffles are mycorrhizal hypogeous fungi, among which several species of the genera \textit{Picoa}, \textit{Balsamia}, \textit{Tuber}, \textit{Tirmania} and \textit{Terfezia} are included. They are of considerable interest for ecological, agroforestry and commercial purposes. Their ecological value is derived from their position in arid ecosystems as symbiotic mycorrhizal fungi associated with annual and perennial species of the genera \textit{Cistus} and \textit{Helianthemum}. The introduction of desert truffle cultivation into dry environments is of strong agroforestry interest since it is a useful way of exploiting lands which have been regarded as unproductive; their introduction would help to improve the social and economical level of these dry regions. In addition, the host plants are xerophytic species characteristic of semiarid environments, and their plantation could help preserve lands from the ravages of erosion [1]. Its edible ascocarps are rich in fiber, proteins, vitamins, and minerals [2], with high commercial value.

Our group has taken \textit{Terfezia claveryi-Helianthemum almeriense} as a model system to study the process of mycorrhization [1,3].

Adverse physical and biochemical changes in \textit{T. claveryi} ascocarps can affect their flavour, colour and texture, lowering their acceptability and nutritional value. To understand these changes, information is required about enzymes such as tyrosinase and lipoxygenase (LOX), that are involved in different oxidative and reductive processes. These oxidases have been purified, characterized and localized within \textit{T. claveryi} ascocarps.

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase which uses molecular oxygen to catalyse two different reactions: the oxidation of monophenols (monophenolase or cresolase activity) [4] and their subsequent oxidation to \(\text{O-quinones (diphenolase or catecholase activity)}\) [5] (Figure 1). The \(\text{O-quinones thus generated polymerise to form melanin through a subsequent series of enzymic and non-enzymic reactions. Tyrosinase is responsible for the undesired enzymatic browning of mushrooms that takes place during senescence or as a result of damage during post-harvest handling. In addition, melanin synthesis in fungi has been correlated with differentiation of reproductive organs and}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{Figure1.png}
\caption{Schematic representation of monophenolase and diphenolase activities catalysed by tyrosinase.}
\end{figure}

Fig. 1. Schematic representation of monophenolase and diphenolase activities catalysed by tyrosinase.
spore formation, the virulence of pathogenic fungi, and tissue protection after injury (see references in [5]). One unusual and intriguing characteristic of this enzyme is its ability to exist in an inactive or latent state, as well as in both forms simultaneously in many sources. One of the few fully latent tyrosinases which has been characterized to date is *T. claveryi* tyrosinase, whose activity can be measured only after activation with different agents. Monophenolase and diphenolase activities of this latent enzyme have been studied both in young and mature ascocarps and the effect of different inhibitors has also been studied. In addition, these two activities have been histochemically located within the ascocarp [4,5] and the technique used demonstrated that tyrosinase activation by certain agents is a reversible process [6].

LOX (EC 1.13.11.12) is a nonheme iron-containing enzyme that uses molecular oxygen in the dioxygenation of a fatty acids containing one or more 1,4-Z,Z0-pentadiene systems, giving rise to their corresponding hydroperoxides (Figure 2).

![Fig. 2. Dioxygenation reaction catalysed by LOX with linoleic acid (OD) as substrate. HPOD, linoleic acid hydroperoxide.](image)

The high proportion of polyunsaturated fatty acids present in *T. claveryi* ascocarps makes lipid rancidity the main factor limiting its storage life, since lipid peroxidation gives rise to unpleasant odours and tastes which lead to consumer rejection. Spoilage caused by oxidative rancidity can be accelerated by enzymes such as LOX. Hydroperoxides produced by this enzyme decompose to form volatile aroma compounds, which are perceived as off-flavours [7]. In addition, the free radicals formed during lipid oxidation may also lead to a reduction in nutritional quality by reacting with vitamins, especially vitamin E, which is lost from foods during its action as an antioxidant. In some plant foods LOX may also contribute to flavour [8]. Despite its importance, there is very little information on LOX from edible fungi.

2 MATERIALS AND METHODS

**Fungal Material.** Ascocarps of *T. claveryi* (Fig. 3) 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.

![Fig. 3. *Terfezia claveryi* ascocarps. Bar = 2.5 cm.](image)

**Extraction of Tyrosinase and LOX.** Pieces of *T. claveryi* ascocarps, containing both peridium and gleba, were suspended in 0.1M 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 15000 g for 20 min. This supernatant was subjected to temperature phase partitioning by adding Triton X-114 (TX-114) 20% (w/v) at 4°C, so that the final detergent concentration was 12% (for tyrosinase) and 8% (for LOX). The mixture was kept at 4°C for 5-10 min and then warmed to 37°C in a thermostatic bath until 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 (Figure 4) was used as the enzymatic extract in the case of tyrosinase and subjected to a second temperature phase partitioning with TX-114 (final detergent concentration 6%) in the case of LOX.

![Fig. 4. Scheme of phase partitioning using TX-114. See text for further details.](image)

**Purification of LOX.** The buffer of the second phase-partitioning supernatant was changed to 50 mM sodium phosphate buffer at pH 5.5. Aliquots of this extract were
loaded onto a 1 mL Resource S column connected to an Äkta purifier (GE Healthcare, Barcelona, Spain) and equilibrated with 50 mM 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. Samples containing LOX were stabilized by adding TX-100 (final concentration 0.04% v/v). Aliquots containing LOX activity were mixed, loaded on the same column and equilibrated with 50 mM sodium phosphate buffer pH 5.5 containing 0.04% TX-100 and 1M NaCl. The elution buffer consisted of 50 mM sodium phosphate pH 5.5 buffer containing 0.04% TX-100 and 1M NaCl.

Enzyme Activity. DiphenoInhibition of Tyrosinase. It was determined spectrophotometrically using L-DOPA as substrate and recording dopachrome production at 475 nm. The standard reaction medium consisted of 2.5 mM SDS, 10 mM L-DOPA in 0.1 M phosphate buffer pH 5.5.

Characterization of Tyrosinase Inhibitors. The reaction medium consisted of 2.5 mM SDS, 0.07 unit of tyrosinase, 1mM L-DOPA and different concentrations of inhibitors.

Polarographic Determination of LOX Activity. Substrate specificity and the pH optimum were determined by measuring oxygen consumption with a Clark-type electrode (Hansatech Lte., Norfolk, UK). To determine the substrate specificity, 1-mL samples consisting of 180 µM substrate in 0.1M phosphate buffer at pH 7.0 were vigorously shaken before use so that they would become air-saturated. They were then transferred to the oxygraph chamber where the reaction was started by adding the enzyme.

Characterization of LOX Inhibitors. The effect of different chemicals on the LOX activity was determined spectrophotometrically. The assays were conducted using 0.1M borate buffer at pH 10.0 to avoid interferences because of the turbidity of the fatty acids. The antioxidants were solubilized in ethanol, except sodium ascorbate, which was prepared in 0.1M sodium phosphate buffer at pH 7.0. The reaction medium contained 0.5mM inhibitor and 180 µM linoleic acid (LA); the reaction was started by adding the enzyme. The control assays were carried out using ethanol without inhibitor.

Analysis of LOX Products. For product analysis LOX was incubated with LA in 0.1 M phosphate buffer (pH 7.0). The products were extracted as described in [9] and analysed by HPLC on a ChromSpher Si column (250 * 4.6 mm), with a 5 µm typical particle size, using hexane/2-propanol/AcH (100:1.6:0.1). The products of T. claveryi were identified comparing their retention time with standards of 13-HPMA and 2-HPMA. For further details see [9].

Histochemical Localization of Tyrosinase. 10-µm-thick sections of both young and mature ascocarps were obtained using a Cryostat Reichert-Jung model 2700 Frigcut. The localization of monophenolase and diphenoInhibition of Tyrosinase. It was determined spectrophotometrically using L-DOPA as substrate and recording dopachrome production at 475 nm. The standard reaction medium consisted of 2.5 mM SDS, 10 mM L-DOPA in 0.1 M phosphate buffer pH 5.5.

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Fig. 5. Purification of LOX from *T. claveryi* ascocarps. A total of 1 mL of TX-114 supernatant was loaded on a Resource-S column (1mL) 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. The area where LOX is eluted is amplified in B.

**Activation of Tyrosinase.** One unusual and intriguing characteristic of tyrosinase is its ability to exist in an inactive or latent state. Tyrosinase can be released from latency or activated by a variety of treatments of agents [13] 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 immature ascocarps. To detect any catecholase activity, the presence of SDS or trypsin in the reaction medium was necessary. The effect of SDS concentration using L-DOPA as substrates is shown in Figure 6. The enzyme remained inactive until the concentration of SDS in the reaction medium was above 0.5 mM. A sharp increase in activity was observed until the critical micelle concentration for the detergent (3.5 mM at pH 6.0) was reached, with half-maximal activation occurring at 0.9 mM. This sigmoidal response is in agreement with the results reported by other authors, who suggested that the amount of SDS needed for activation is related to the maximum number of SDS monomers in solution [14], thus suggesting a conformational change associated with binding of the SDS monomers [13]. Further information on the effect of trypsin and SDS on *T. claveryi* tyrosinase is presented in [6].

![Fig. 6. Percentage of activation of *T. claveryi* tyrosinase with different concentrations of SDS. The reaction medium contained 0.5mM L-DOPA in 0.1 M phosphate buffer pH 5.5.](image)

**Histochemical Localization of Tyrosinase.** 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. However, the application of this method has been hampered by endogenous substrates, inhibitors, and phenolics which can inactivate enzymes [15]. Thus, a reliable method for locating enzymes in whole tissue sections would have considerable application to fungal biochemistry.

Both monophenolase and diphenolase activities of tyrosinase have been localized in the peridium, hypotecium, asci, spores and ascogenic hyphae of the mature ascocarps of *T. claveryi* (4-6). No reaction was observed when the sections were incubated with tyrosinase without SDS of both mature and young ascocarps, or with control sections incubated with only buffer (Figure 7).

![Fig. 7. Localization of tyrosinase in sections of *T. claveryi* mature ascocarps. The sections were incubated in L-DOPA containing SDS (A) or in 0.1 M phosphate buffer pH 5.5 (B). as, ascospores.](image)

The co-localization of monophenolase and diphenolase activities of *T. claveryi* tyrosinase confirms the bifunctional character of this enzyme. The reversibility of SDS activation was clearly confirmed using sections of *T. claveryi* ascocarps since when sections were preincubated...
Characterization of the Diphenolase Activity of Tyrosinase. The pH profiles of catecholase using L-DOPA as substrate indicate that the enzyme was active between pH 3.5 and pH 7.0, showing a maximum between pH 5.5 and 6.0 (data not shown). The kinetic parameters ($V_{max}$ and $K_m$) of L-DOPA oxidation were studied at the optimum pH. $K_m$ and $V_{max}$ were calculated by non-linear regression fitting [16] of the experimental points to the equation of Michaelis-Menten. The values obtained for $K_m$ gave a value of 12 mM.

The effect of specific inhibitors (kojic and cinnamic acid, mimosine and tropolone) on the catecholase activity of *T. claveryi* tyrosinase, using L-DOPA as substrate, was also analysed. This study is particularly interesting because the inhibition of tyrosinase activity might have an influence on the mycelium growth [17]. Table 1 shows the percentage of residual activity obtained with different concentrations of these compounds. Among these, the substrate analogue tropolone was the most effective inhibitor. Kojic acid, a fungal metabolite, was also a potent inhibitor of *T. claveryi* tyrosinase, and only 4% of the residual activity could be detected when a concentration of 0.9 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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Residual activity (%)</th>
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<tbody>
<tr>
<td>Tropolone</td>
<td>0.01</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>0.01</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>28</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>0.01</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>4</td>
</tr>
<tr>
<td>L-mimosine</td>
<td>0.01</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>8</td>
</tr>
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</table>

Table 1. Effect of different inhibitors on diphenolase activity of tyrosinase.

Characterization of LOX. The pH profile obtained presents the maximum activity at pH 7.0 (Figure 8) and is typical of other LOX.

When the substrate specificity of the purified LOX enzyme was investigated, the highest relative enzymic activity was obtained using linoleic acid as the substrate (100%), followed by linolenic acid (91%). The lowest relative LOX activity (32%) was exhibited toward γ-linolenic acid. Since linoleic acid represents 45.4% of total fatty acids within raw truffles while linolenic acid represents 5.8% [2], these data suggest that endogenous linoleic acid is the preferred substrate for LOX from *T. claveryi* ascocarps.

Fig. 8. Effect of pH on the LOX activity. The reaction medium (1 mL) contained 0.1 M phosphate buffer pH 6.0-7.6 (squares), and 0.1 M borate buffer pH 7.6-10.0 (triangle), LOX and 1 mM linoleic acid.

Effect of Different Compounds on LOX Activity. The effect of different compounds (final concentration of 0.5mM) on the LOX activity was also studied [18]. The results presented in Table 2 indicated that nordihydroguaiaretic acid (NDGA), a catecholic antioxidant, is an effective inhibitor, decreasing its activity by 94% at 0.5 mM. Kemal and coworkers [19] demonstrated that this compound inhibited soybean LOX by reducing the catalytically active Fe$^{3+}$ to the catalytically inactive Fe$^{2+}$.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>49</td>
</tr>
<tr>
<td>BHT</td>
<td>62</td>
</tr>
<tr>
<td>BHA</td>
<td>44</td>
</tr>
<tr>
<td>Esculetin</td>
<td>28</td>
</tr>
<tr>
<td>NDGA</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2. Effect of different inhibitors on LOX activity.

Analysis of Reaction Products. The elution profile of the products obtained by incubating linoleic or linolenic acids with LOX showed one major peak which corresponded to 13-Z,E-HPOD. According to [20], the synthesis of a single specific hydroperoxide from free fatty acid substrates is related to the formation of biological mediators of signalling molecules. In plants, these hydroperoxides serve as a substrate for enzymes such as hydroperoxide lyase, peroxygenase, hydroperoxide reductase etc. Some of these fatty acid derivatives represent biological signals, which do not require the prior activation of genes [21]. Although many authors have reported the induction of plant LOX by fungi [22], the role of fungal LOX in mycorrhizal symbiosis has never been studied.
4 ACKNOWLEDGEMENTS

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