Characterization of tyrosinase and accompanying laccase from

*Amorphophallus campanulatus*

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Tyrosinase and laccase activities were detected in the corm of *Amorphophallus campanulatus* after extraction with ethanol followed by ammonium sulphate precipitation (20-60%) and dialysis against 10 mM Na₂HPO₄ buffer at pH 7.0. Tyrosinase was found to be the predominant enzyme exhibiting mono- and di-phenolase activities, specificity for l-DOPA as substrate, optimum pH being 6.0, optimum temperature at 40°C and Kₘ at 1.05 mM. Laccase showed substrate specificity for p-phenylenediamine (p-PD), Kₘ at 2.7 mM, optimum pH being 5.0 and was inactivated above 40°C. Three isoforms of tyrosinase were detected on SDS-PAGE with apparent molecular mass ~127, 31 and 27 kDa respectively. On staining sections of *A. campanulatus* with l-DOPA as substrate and 3-methyl benzothiazolinone hydrazone (MBTH) for colour development, tyrosinase was detected in the intercellular spaces of the plant tissue. The cytosolic region did not show any colour indicating the absence of the enzyme.

The most abundant in terms of occurrence amongst the polyphenol oxidases (PPOs), is the enzyme tyrosinase (monophenol, o-diphenol: oxygen oxidoreductase, EC 1:14:18:1), while other enzymes falling under this class include laccase (p-diphenol: oxygen oxidoreductase, EC 1:10:3:2) and catechol oxidase (o-diphenol: oxygen oxidoreductase, EC 1:10:3:1) respectively¹². In higher plants the PPOs are thought to be involved in the defense against insects and microorganisms by forming a resistant layer of melanin pigments on the surface of the damaged tissues, rendering it a brownish black colour³. This browning effect caused by the melanin compounds is undesirable in case of canned food products as it leads to the loss of food quality and market rejection⁴⁵. On the other hand, it is found to be desirable in the production of tea, coffee and cocoa beverages for the development of colour and flavors⁶. Tyrosinases have been characterized from various plant sources⁴⁷ and are known to utilize a variety of mono- and di-phenol substrates, although the preferred substrates for assaying the enzyme activity include tyrosine, dopa and catechol. The enzyme is distinguishable from other PPOs especially the laccase, using specific inhibitors such as SHAM, tropolone, cinnamic acid, 2,3-naphthalenediol, and 4-hexylresorcinol, which affect laccase activity minimally⁸.

The tuberous roots of *Amorphophallus campanulatus*, commonly known as ‘Soran’ are used in India in curries and pickles and are ascribed with therapeutic activities against piles and dysentery⁹. When the corm of this tuber is cut open it gets coated with a thick protective layer of brown pigment which is probably indicative of high levels of PPO activities associated with it. The tyrosinase extracted from this source has been successfully used in the fabrication of biosensors by us recently¹⁰¹¹. We describe here the extraction and characterization of tyrosinase from *A. campanulatus* along with the elucidation of the laccase activity associated with it, which contributes to the browning reaction and the histochemical localization of the tyrosinase.

**Materials and Methods**

**Materials**

The corm of *A. campanulatus* was purchased freshly from the local market. The chemicals including l-DOPA, tyrosine, p-PD, MBTH and SHAM were obtained from Sigma Chemicals, UK. Catechol, 4-methyl catechol and CTAB were products of SRL Chemicals (India). The reagents for electrophoresis were procured from BioRad (India). All other chemicals were of analytical grade.
Preparation of enzyme

Peeled corm of *A. campanulatus* (20 g) was homogenized in chilled 0.1 M Na2HPO4 buffer (pH 7.0) filtered through four layers of cheesecloth and was centrifuged at 2500 rpm for 7 min at 4°C. The insoluble matter was precipitated out from the supernatant by the addition of 12.5% (v/v) chilled ethanol and the supernatant was subjected to ammonium sulphate fractionation between 20 to 60% saturation at 4°C. The precipitate obtained at 60% saturation was re-suspended in the extraction buffer, dialyzed against 10 mM Na2HPO4 buffer (pH 7.0) and used as the enzyme source.

To solubilize the membrane bound tyrosinase the supernatant after centrifugation (2500 rpm) was either repeatedly frozen at -70°C and thawed at room temperature or was incubated with 1% solution of Triton X-100 or SDS or Brij-35 overnight at 4°C temperature or was incubated with 1% solution of sodium deoxycholate for 12 h. The enzyme was centrifuged at 2500 rpm for 7 min at 4°C. The supernatant after centrifugation (2500 rpm) was either repeatedly frozen at -70°C and thawed at room temperature or was incubated with 1% solution of Triton X-100 or SDS or Brij-35 overnight at 4°C temperature or was incubated with 1% solution of sodium deoxycholate for 12 h. The enzyme was centrifuged at 2500 rpm for 7 min at 4°C.

Optimum pH of tyrosinase and laccase

Citrate-phosphate buffers (0.1 M) in the pH range of 4.0-6.0 and Na2HPO4 buffer in the pH range 5.8-7.5 were used. Tyrosinase activity was monitored using 2 mM L-DOPA and 4 mM tyrosine as substrates while laccase activity was measured employing 4 mM *p*-PD and L-DOPA as substrates respectively.

Optimum temperature of tyrosinase and laccase

The assay mixture containing 0.48 mg of the enzyme was incubated at different temperatures from 15-70°C for 5 min followed by the addition of respective substrate (2 mM L-DOPA for tyrosinase and 4 mM *p*-PD for laccase).

Effect of inhibitors

Effect of varying concentrations of the inhibitors on enzyme activity was studied. In the case of tyrosinase, SHAM (50-150 μM) was added to L-DOPA as substrate. In the case of laccase, CTAB (0.5-1.5 mM) was used as inhibitor and *p*-PD as substrate. The inhibition was measured as the decrease in the initial velocity.

The effects of specific inhibitors like SHAM (for tyrosinase) and CTAB (for laccase) were determined using the final concentrations of 1 mM and 0.100 mM for SHAM and CTAB, respectively. The rate of reaction and % inhibition were calculated from the linear portion of the absorbance *versus* time plots.

**Enzyme assay**

**Monophenolase activity**

The reaction mixture contained 4 mM tyrosine, 1.6 mM MBTH and 0.1 mM L-DOPA with 0.48 mg of enzyme solution in 1 ml of 0.1 mM Na2HPO4 buffer (pH 6.0) containing 2% DMF at 37°C. The enzyme activity was monitored spectrophotometrically at 505 nm.

**Diphenolase activity**

The reaction mixture contained 0.48 mg of the enzyme solution in 1 ml of 0.1 mM Na2HPO4 buffer (pH 6.0) and different substrates such as 2 mM L-DOPA (475 nm), 4 mM DL-DOPA (475 nm), 2 mM catechol (410 nm) and 4 mM 4-methyl catechol (410 nm), respectively. The enzyme activity was measured spectrophotometrically at the wavelength indicated for each substrate.

**Laccase activity**

The reaction mixture contained 0.48 mg of the enzyme in 1 ml of 0.1 M citrate-phosphate buffer (pH 5.0), 4 mM L-DOPA (475 nm) and 4 mM *p*-PD (410 nm) as substrates respectively. The enzyme activity was measured spectrophotometrically at the wavelength indicated for each substrate. The rates of reactions and *k* values were calculated from the linear region of absorbance *versus* time curves. The enzyme activity was defined in the unit of Katal, which is the amount of enzyme used in the conversion of one μmole of substrate per second.

SDS-PAGE was carried out in 10% polyacrylamide gel in Tris-glycine buffer (pH 8.3) system by the method of Laemmli16 as modified by Angleton and Flurkey17 for the partially denaturing conditions. The sample was prepared in 0.1% SDS avoiding 2-mercaptoethanol and heat treatment. Tyrosinase was located by activity staining. After electrophoresis the gels were soaked in 10 mM Na2HPO4 buffer (pH 6.0) for 10 min and cut into vertical strips, followed by incubation in the same buffer containing 2 mM L-DOPA and 4 mM MBTH at 37°C in the presence and absence of 150 μM of SHAM inhibitor. The isoenzyme patterns were recorded using Kodak film.
Histochemical studies

Fresh corm sample was peeled and cut into the sections of 6 to 8 microns using rotary microtome (Richard Tung, UK) under ice-cold conditions. The sections were stained with L-DOPA and MBTH solutions in Na₂HPO₄ buffer (pH 6.0), just prior to the microscopic evaluation. Microphotographs were taken on Zeiss Axioptan microscopic assembly (Germany).

Results and Discussion

The major phenol oxidase in the enzyme extract of *A. campanulatus* was found to be tyrosinase although laccase activity was also observed. The simple extraction procedure employed helped to preserve both monophenolase and diphenolase activities in the enzyme extract. Loss of monophenolase activity during purification process involving removal of phenolics has been reported by some workers. Loss of monophenolase activity during purification process involving removal of phenolics has been reported by some workers. Monophenolase activity was found to be five and twelve times lower than laccase and diphenolase activity, respectively.

Effect of substrates

Tyrosinase activity in the corm extract was highest with L-DOPA as a substrate (Table 1). The apparent $k_m$ value of 1.05 mM obtained (Fig. 1) from the Lineweaver-Burk double reciprocal plots of initial velocity against the substrate concentrations (L-DOPA) is similar to that reported for tyrosinases from other plant sources. Laccase activity was higher with p-PD than with L-DOPA as substrate. The $k_m$ value for p-PD was 2.7 mM and for dopa it was 28.5 mM, indicating that p-PD is the preferable substrate for laccase.

Effect of inhibitors

Tyrosinase and laccase activities were determined based on their selectivities towards *ortho*- and *para*-diphenoloxidases. Specific inhibitors, viz. SHAM and CTAB, were used as suggested by Kumar and Flurkey. It was observed that ~12 μM SHAM competitively inhibited tyrosinase activity by 50%, L-DOPA being the substrate. CTAB (1 mM) caused mixed-type of inhibition on laccase activity resulting in 50% inhibition with p-PD as the substrate (Fig. 2). CTAB (1.5 mM at which laccase activity was inhibited more than 80%) had negligible effect on tyrosinase activity (Fig. 1).

Effect of pH

The optimum pH for tyrosinase was observed to be around 6.0 although stable activity was evident in the pH range of 6.0 to 7.0. The optimum pH for the laccase activity using *p*-PD and L-DOPA as

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (katal)</th>
<th>$k$ (mM)</th>
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<tbody>
<tr>
<td>L-DOPA</td>
<td>14010</td>
<td>1.05</td>
</tr>
<tr>
<td>dl-DOPA</td>
<td>2340</td>
<td>4.00</td>
</tr>
<tr>
<td>Catechol</td>
<td>5725</td>
<td>3.13</td>
</tr>
<tr>
<td>4-Methylcatechol</td>
<td>5150</td>
<td>5.26</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>130</td>
<td>1.33</td>
</tr>
</tbody>
</table>

Fig. 1—Lineweaver-Burk double reciprocal plot of initial velocity against substrate concentration for the inhibition of *A. campanulatus* tyrosinase by ( ), CTAB, 1.5 mM; (▲), SHAM, 100 μM; (□), No inhibitor [Reaction mixture included 0.48 mg protein, 2 mM L-DOPA, in 0.1 M Na₂HPO₄ buffer, pH 6.0]
substrates was 5.0 and 7.0, respectively (Fig. 3). These are consistent with the pH profiles reported for fungal laccases.

There was no lag period for monophenolase activity at pH 6.0, using L-tyrosine as the substrate in the presence of catalytic amount of L-DOPA (0.1 mM). The activity was found to increase with increase in pH up to 7.0 after which it dropped considerably giving a bell-shaped curve (Fig. 4). This observation is consistent with the properties reported for monophenolase activity of grape polyphenol oxidase by Sanchez-Ferrer et al.

**Effect of temperature**

As shown in Fig. 5, the enzyme extract of *A. campanulatus* showed minor losses in the tyrosinase activity even at 45 to 50°C. Laccase activity underwent rapid inactivation when pre-incubated for 5 min at temperatures > 40°C. The energy of activation for tyrosinase was found to be $E_a = 11,502$ cal/mole at 40°C. The energy of activation for denaturation of 50% of tyrosinase activity was obtained as $E_a = 7,145$ cal/mole at 60°C.

**SDS-PAGE**

Partially denaturing SDS-PAGE of the enzyme extract of *A. campanulatus* showed three isoforms (Fig. 6) with $R_f$ values of 0.04, 0.54 and 0.59 respectively on staining with L-DOPA and MBTH. A parallel gel was stained with Coomassie Blue R250 for protein detection. Apparent molecular masses of the isoforms calculated by the method of Weber and Osborn were found to be ~127, 31 and 27 kDa, respectively.

The enzyme extract contained both tyrosinase and laccase activities, wherein the former could be completely inhibited by SHAM. In order to differentiate between tyrosinase and laccase isoforms after the electrophoretic run, the gels were stained for the tyrosinase activity in the presence of SHAM.
Fig. 5—Effect of temperature on the initial velocity of tyrosinase and laccase activities of *A. campanulatus* [Details are mentioned under Materials and Methods]

(Fig. 6a) which revealed only one band with less color intensity and of R<sub>f</sub> value, 0.48. This indicated that isoforms with R<sub>f</sub> values 0.04, 0.54 and 0.59 were completely inhibited by SHAM and correspond to tyrosinase, while the band having R<sub>f</sub> of 0.48 represented the laccase activity.

**Histochemical studies**

In order to localize the tyrosinase enzyme *in situ*, sections of *A. campanulatus* were stained with the specific substrate (i.e. L-DOPA) and scanned microscopically. Figs 7a and b show the microscopic pictures of these sections before and after staining with L-DOPA and MBTH solutions. It was observed that L-DOPA reacted with tyrosinase *in situ* forming the dopaquinone intermediate, which instantaneously reacted with MBTH forming a pink colored complex at the site of the enzyme. Tyrosinase was thus found
to be localized in the intercellular spaces of the plant tissue, probably attached to the cell wall membrane. The staining did not appear continuously throughout the tissue but appeared as patches. When monitored microscopically over a period of time, the cytosolic region of the cell remained colourless indicating the absence of the enzyme.

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