PURIFICATION OF LACCASE

5.1 Introduction

Laccase is an enzyme which is widely distributed in many genera of white rot fungi (Bollag et al. 1984, Mayer 1987, Sannia et al. 1986, Thurston 1994, Youn et al. 1995). A number of laccase isozymes have been isolated and purified from Pleurotus spp. (Munoz et al. 1997, Palmieri et al. 1993, Sannia et al. 1986, Youn et al. 1995). Pleurotus florida produces two laccase enzymes (L1 and L2) within which L2 is associated with the vegetative growth of the fungus. In this analysis, the purification of L2 enzyme from culture filtrate of P. florida was carried out.

5.2 Materials and methods

5.2.1 Enzyme production

The P. florida strains isolated from the foot hills of western ghats, Tamil Nadu and Karnataka, India, were maintained in PDA medium (Shanmugam et al., 2005). The mushroom fungus was grown in potato dextrose yeast extract (PDY) medium for 5 days. The culture supernatant was obtained by centrifugation (21,000 x g, 30 min). About 1000 ml of culture filtrate was concentrated by lyophilisation and this concentrate was used for the enzyme purification.

5.2.2 Ammonium sulphate precipitation

From the culture filtrate concentrate, about 100 ml was subjected to 0–80% ammonium sulphate precipitation by dialysis. The resulting solution in the bag was subjected to centrifugation at 80,000 x g for 1 h and the protein precipitate was collected. The precipitated pellet was then dissolved in the buffer (10 mM sodium acetate buffer, pH 5.0) and subsequently dialysed against the same buffer using a dialysis membrane with 10 Kda cut-off (sigma). The resulting extract was used for further chromatographic analysis.
5.2.3 Laccase purification

Laccase was purified from the culture supernatant of *P. florida* by anion exchange chromatography and gel permeation chromatography. 5ml of the enzyme extract was applied to a DEAE – Sephadex (A-50) column (22 ×220mm) equilibrated with buffer A (10mM sodium acetate buffer, pH 5.0) and was eluted with a linear gradient of 0.1–0.6 M NaCl. Upon this, two laccase peaks were obtained and the fractions of the second peak were concentrated to 1 ml and dialysed against buffer B (100mM sodium acetate buffer, pH 5.0). The resulting sample was then applied to a Bio Gel P – 200 column (16 × 650 mm) for further purification. Active fractions obtained were pooled together, concentrated and once again dialysed against buffer B and the resulting solution was subjected to electrophoresis.

5.2.4 Polyacrylamide gel electrophoresis (PAGE)

5.2.4.1 Native PAGE

Native PAGE was carried out according to the method of Laemmli (1970) with some modifications. 5 % stacking gel (6.8 ml H₂O, 1.7ml 30% acrylamide (Bio-Rad), 1.25ml 1M Tris (pH 6.8), 0.1ml APS (freshly prepared), 0.01ml TEMED) and 10% resolving gel (4.01ml H₂O, 3.34 ml 30% acrylamide (Bio-Rad), 2.5ml 1.5M Tris (pH 8.8), 0.4ml 10%APS (freshly prepared). Freshly prepared running buffer (25mM Tris, 192mM glycine (pH 8.3) was used. Samples (30μg protein/well) were loaded and electrophoresed at a constant voltage of 200V for thirty minutes in a Bio-rad mini electrophoresis apparatus. After electrophoresis the gel was stained for laccase activity with 0.38mM guaiacol in 50 ml of 100mM sodium acetate buffer pH 6.0 at room temperature.

5.2.4.2 SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli (1970) with some modifications. 5 % stacking gel (3.4 ml H₂O, 0.85 ml 30% acrylamide (Bio-Rad), 0.32ml 1M Tris (pH 6.8), 0.05ml 10% SDS and 0.1ml 10% APS (freshly prepared), 0.025ml TEMED) and 12% resolving gel (3.2ml H₂O, 4.0 ml 30% acrylamide (Bio-Rad), 2.5ml 1.5M Tris (pH 8.8), 0.1ml 10%SDS and 0.1ml 10%APS (freshly prepared).
Freshly prepared running buffer (25mM Tris, 192mM glycine and 0.1% SDS (pH8.3) was used. Samples (10-50μg protein) was mixed with sample loading buffer(6x) final concentration (120mM Tris, (pH 6.8), 30%(v/v) glycerol, 4% SDS, 4% mercaptoethanol and 0.02% bromophenol blue) and boiled for 3 minutes and electrophoresed at a constant 200V for 30 minutes in a Bio-rad mini electrophoresis apparatus. For molecular weight calibration, broad range molecular weight markers (Bio-rad) (Myosin 200KDa, β-galactosidase 116.25 KDa, phosphorylase b 97.4 KDa, Bovine serum albumin 66.2 KDa, Ovalbumin 45KDa, carbonic anhydrase 31KDa, Lysozyme 14.1 KDa) were used as the protein standards and prepared according to the manufacturers instructions.

5.2.4.3 Coomassie Staining

The gel was stained with commassie blue R-250(Sigma) (2g coomassie blue, 300ml methanol, 100ml glacial acetic acid and 400ml deionised water) for 2 hours and destained overnight in half strength destaining solution (150ml methanol, 50ml glacial acetic acid and 600ml deionised water). Further destaining was carried out with deionised water.

5.2.5 Laccase activity assay

The activity of laccase was determined as described in Chapter 2

5.2.6 Enzyme kinetics

The apparent Km values were determined from Lineweaver – Burk plots using guaiacol (0.15mM – 0.8 mM) and o-dianisidine (0.5mM – 7.0 mM). The pseudo – first order rate constants were obtained using the relation K = Vmax / Km

5.3 Results and discussion

Upon ammonium sulphate precipitation, Ion exchange and gel permeation chromatography based purification of L2 isozyme secreted extracellularly by P. florida, the purification fold was calculated. It was estimated to be 34.32 with an yield of 7.66% using Biogel P-200 .The enzyme activity, total protein present, its specific activity, purification fold and yield of the purified laccase enzyme are given in (Table 5.1). The 280/600nm absorbance ratio of purified L2 was determined to be 20. The
molecular mass of the native protein was estimated to be 71 kDa by gel permeation chromatography and 70 kDa based on SDS–PAGE.

The pH profile of *P. florida* L₂ laccase showed optimum activity at pH 6.0 and 5.5 using guaiacol and o-dianisidine respectively as the substrates (Fig.5.1 and Fig.5.2). Where as the optimum pH of both the laccase isozymes of *P. eryngii* was found to be 4.0 using ABTS as the substrate (Munoz et al., 1997). The highest activity of *P. ostreatus* laccases in respect of pH profile varied with the changes of substrate (Palmieri et al., 1993, Youn et al., 1995). The variation of optimum pH might be due to different role of substrate protonation in the reaction mechanism (Palmieri et al.,1993). L₂ isozyme of *P. florida* showed wide range of pH stability (pH 5.0 – 7.0) as in other *Pleurotus* sp. (Palmeri et al., 1993).

*P. ostreatus* produced laccase is almost fully active in the temperature range of 40-60°C and showed a half life of 30 min at 60°C (Palmieri et al., 1993). Laccase I and II of *P. eryngii* retained 3 % and 10 % residual activity respectively at 60°C after 30 min incubation (Munoz et al., 1997). However, Youn et al. (1995) have reported that the optimum temperature for laccase L₁ of *P. ostreatus* as 30 – 35°C. Similarly the studies showed that the L₂ laccase enzyme of *P. florida* showed its optimum activity at 50°C which was quite similar to the laccase produced by *P. ostreatus* (Palmieri et al.,1993) (Fig.5.3 and Fig.5.4). Purified L₂ is stable at 40°C for 5h, like that of other fungal laccases (Coll et al., 1993a, Palmieri et al., 1993, Slomczynski 1995). The study also revealed that the L₂ of *P. florida* showed a half life of 20 min at 60°C. Further, Arrhenius plot indicated the activation energy of L₂ as 3.9 kJ/mol of protein whereas it was reported to be 10.7 kJ/mol for *P. ostreatus* laccase by Youn et al., 1995.

Fungal laccases are known to catalyze a wide range of substrates (Eggert 1997, Mayer 1987, Youn et al., 1995) and even the laccase isozymes of the same strain may also show differential substrate affinities (Fukushima and Kirk 1995, Munoz et al., 1997). The *Kₘ* value of L₂ enzyme for guaiacol was estimated to be much higher than *P.
ostreatus laccase (Fig.5.5 and Fig.5.6). The L2 isozyme of *P. florida* showed notable differences in its catalytic efficiencies when substrates were varied (Table 5.2). The \(K_{\text{cat}}/K_m\) values of L2 were determined to be \(2.41 \times 10^6 \text{ mM}^{-1}\text{sec}^{-1}\) and \(1.0 \times 10^5 \text{ mM}^{-1}\text{sec}^{-1}\) with guaiacol and o-dianisidine respectively. All the kinetic parameters of L2 enzyme suggested that it has a higher affinity towards guaiacol than o-dianisidine. Similar kind of observation was reported for laccases of *P. eryngii*, where p-anisidine was preferred as a laccase substrate than guaiacol (Munoz *et al.*, 1997).

### 5.4 Conclusion

From this study, it was found that the purified L2 laccase enzyme produced by *P. florida* has an optimum pH at 6.0 and 5.5 for the substrates guaiacol and o-dianisidine. The optimum temperature for purified laccase activity was found to be 50 °C for both the substrates. The \(K_m\) value for guaiacol and o-dianisidine was found to be 0.38mM and 1.96mM respectively and guaiacol seemed to possess a low \(K_m\), which proved to be a good substrate than o-dianisidine. The \(K_{\text{cat}}/K_m\) values of L2 were determined to be \(2.41 \times 10^6 \text{ mM}^{-1}\text{sec}^{-1}\) and \(1.0 \times 10^5 \text{ mM}^{-1}\text{sec}^{-1}\) with guaiacol and o-dianisidine respectively. Further, Arrhenius plot indicated the activation energy of L2 as 3.9 kJ/mol of protein.

Table 5.1 Purification of laccase enzyme from *P. florida*,

<table>
<thead>
<tr>
<th>Purification step(s)</th>
<th>Total enzyme activity (mg)</th>
<th>Total protein (mg)</th>
<th>Sp.activity (U/mg)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>4000.00</td>
<td>60.00</td>
<td>83.33</td>
<td>1.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Ammoniumsulphate precipitation (0–80%)</td>
<td>1583.00</td>
<td>23.50</td>
<td>109.90</td>
<td>1.32</td>
<td>39.575</td>
</tr>
<tr>
<td>DEAE Sephadex (A-50)</td>
<td>510.62</td>
<td>2.40</td>
<td>347.16</td>
<td>2.08</td>
<td>12.76</td>
</tr>
<tr>
<td>Biogel P-200</td>
<td>306.72</td>
<td>0.175</td>
<td>2860.0</td>
<td>34.32</td>
<td>7.668</td>
</tr>
</tbody>
</table>

* Laccase was assayed with guaiacol as substrate.
Table 5.2. Kinetic parameters of purified L2 laccase enzyme of *P. florida*

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Guaiacol</th>
<th>o-dianisidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$</td>
<td>0.38 mM</td>
<td>1.96 mM</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>0.82 mM sec$^{-1}$</td>
<td>4.91 mM sec$^{-1}$</td>
</tr>
<tr>
<td>$K$</td>
<td>2.15 sec$^{-1}$</td>
<td>2.51 sec$^{-1}$</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>1.27 sec</td>
<td>0.011 sec</td>
</tr>
<tr>
<td>$K_{cat}$</td>
<td>9.16 x 10$^5$ sec$^{-1}$</td>
<td>1.96 x 10$^5$ sec$^{-1}$</td>
</tr>
<tr>
<td>$K_{cat}/K_m$</td>
<td>2.41 x 10$^6$ mM$^{-1}$ sec$^{-1}$</td>
<td>1.0 x 10$^5$ mM$^{-1}$ sec$^{-1}$</td>
</tr>
</tbody>
</table>
Figure 5.1 Effect of pH on laccase activity using guaiacol as substrate

Figure 5.2 Effect of pH on laccase activity using o-dianisidine as substrate
Figure 5.3 Effect of temperature on laccase activity using guaiacol as substrate

Figure 5.4 Effect of temperature on laccase activity using o-dianisidine as substrate
Figure 5.5 Effect of guaiacol concentration on laccase activity

Figure 5.6 Effect of o-dianisidine concentration on laccase activity
Purification of laccase from *Pleurotus florida* - SDS PAGE

Lane 1 - Marker
Lane 2 - Crude filtrate
Lane 3 - Purified laccase

Purification of laccase from *Pleurotus florida* - Native PAGE

Lane 1 - Marker
Lane 2 - Crude filtrate
Lane 3 - Purified laccase