Purification of Enzyme Phenoloxidase from Freshwater Crab, *Barytelphusa cunicularis* and its Kinetic Study

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**Abstract:** Phenol oxidase (EC: 1.14.18.1) was purified from the prophenol oxidase (pro- PO) system of freshwater crab, *Barytelphusa cunicularis*. The column chromatography purification on Sepharose 4B resulted in high specific activity of PO and high purification. The $K_m$ was found to be 1.21mM and $V_{max}$ $16.31 \times 10^{-4}$ by Lineweaver-Burk plot.

**Key words:** Phenol Oxidase • *Barytelphusa cunicularis* • Purification

**INTRODUCTION**

The commission on biochemical nomenclature included the phenol oxidases in 1974 list as monophenol oxygenase EC:1.14.18.1. This is based on the observation that the action of these copper enzymes on monophenols is catalyzed by traces of diphenols and that isotopic marker experiments show the direct incorporation of oxygen from $O_2$ into the diphenol formed. The phenoloxidases (PO) plays an important role in cuticular sclerotization and in defense against pathogens and parasites by insects and crustaceans [1-3]. PO catalyses hydroxylation of monophenols to O-diphenols and the oxidation of O-diphenols to quinines. The quinines take part in sclerotization and tanning of the cuticle and serve as precursors for the synthesis of melanin. PO is synthesized in arthropods as an inactive zymogen, prophenol oxidase (proPO), which is activated by proteolytic cleavage at a specific site near the proteins amino terminus. In the present study an attempt was made to purify and to study the kinetic properties of the PO enzyme from freshwater crab, *Barytelphusa cunicularis* (West- Wood).

**MATERIALS AND METHODS**

The freshwater crab, *B. cunicularis* were procured from NathSagar dam, Paithan, near Aurangabad. The crabs were maintained at 20°C and fed *ad libitum*. Only male crabs of intermoult stages were used for the purification of PO. All reagents used were of analytical reagent grade, unless other-wise indicated. Sephadex and Sepharose were from Pharmacia, Sweden.

**Preparation of Hemocyte Lysate Supernatant (HLS):** An anticoagulant buffer (citrate-EDTA buffer, pH 4.6) was used for isolating intact hemocytes from crustacean hemolymph. 400µl of anti-coagulant buffer was injected in the coxa of the 4th leg and allowed to circulate in the circulatory system of the crab for three minutes. After three minutes the hemolymph was collected from the chelicerae. The hemolymph was diluted and the diluted sample was centrifuged at 800xg for 15 minutes. The resulting pellet was washed twice with 2ml of CAC buffer (0.01M sodium cacodylate buffer pH 7 containing 5mM calcium chloride and 0.25M sucrose) and homogenized in glass homogenizer. The cell homogenate was centrifuged at 28000xg for 15 minutes and the resulting supernatant was used as a source of prophenol oxidase system. All experiments were carried out at 4°C.

**Enzyme Purification:** The HLS was subjected to salt fractionation at 30-50% ammonium sulfate at pH 7.5 and temperature 4°C. The precipitate were removed by centrifugation and were re-dissolved in CAC buffer, pH 7.5. This solution was then subjected to dialysis in 2L CAC buffer for overnight in a dialysis tube. The dialyzed protein solution was analyzed for PO, after centrifuging at 14000xg for 30 minutes. The salt free solution was passed through Sephadex (G 75-120) and Sepharose 4B column.
which was previously equilibrated with CAC buffer, pH 7.5. The maximum purity in both the columns were analyzed and the procedure yielding the highest purity was used for enzyme assay. The column was eluted with elution buffer (100mM calcium chloride, 10mM NaCAC, pH 6.8) at a flow rate of 1.5ml/minute. The fraction containing PO were pooled and again loaded in Sepharose 4 B column equilibrated with CAC buffer the PO fraction was eluted in fraction 26. This fraction was used for further study.

**Enzyme Assay:** PO was assayed by measuring the formation of dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) from L-dihyroxy phenylalanine (L-DOPA) at 20°C. The assay procedure was followed as described by Leonard et al. [4]. Under the conditions of the assay, the rate of dopachrome formation was measured at 490nm in spectrophotometer.

**PO Unit:** The unit of PO activity was defined as the amount of enzyme which produces an absorbance increase of 1.0 at 490nm.

**Enzyme Kinetics:** Michaelis-Menten constant ($K_m$) was determined by incubating PO enzyme (purified by column chromatography on Sepharose - 4B) and substrate (L-DOPA; 1-6mM). The rates of the initial absorbance changes were monitored at 490 nm. The $K_m$ value was analyzed by Lineweaver-Burk plot [6]. The Microsoft Excel was used to plot and analyze kinetic data.

**Protein Determination:** During the course of isolation of PO enzyme the protein concentration in each steps were determined by Lowry’s method as described by Lowry et al. [5].

**RESULTS AND DISCUSSION**

The PO purification is shown in Table 1. The highest PO purity was found in elution fraction 29 in sepharose 4B column chromatography with a specific activity of 2. The purified PO was examined for its ability to oxidize L-DOPA at various substrate concentration. The PO showed a $K_m$ value of 1.21mM determined by Lineweaver-Burk plot (Fig. 1). The study showed a inhibitory effect of PO at

![L.B. Plot of Substrate concentration (L-DOPA) Vs Velocity of enzyme reaction](image)

Table 1: Purification procedure for PO

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Homogenate</th>
<th>Vol. (ml)</th>
<th>Concentration</th>
<th>Total Units</th>
<th>Protein Concentration mg/ml</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>HLS</td>
<td>9.0</td>
<td>0.0525</td>
<td>0.470</td>
<td>104.000</td>
<td>0.00051</td>
<td>100.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2.</td>
<td>Ammonium Sulphate 30-50%</td>
<td>8.0</td>
<td>0.0860</td>
<td>0.684</td>
<td>153.600</td>
<td>0.00056</td>
<td>145.50</td>
<td>1.09</td>
</tr>
<tr>
<td>3.</td>
<td>Dialysis</td>
<td>9.5</td>
<td>0.0733</td>
<td>0.695</td>
<td>64.000</td>
<td>0.00115</td>
<td>147.80</td>
<td>2.24</td>
</tr>
<tr>
<td>4.</td>
<td>Sephadex G 75-120 (14)*</td>
<td>2.0</td>
<td>0.0050</td>
<td>0.010</td>
<td>0.003</td>
<td>1.67</td>
<td>2.10</td>
<td>3252.80</td>
</tr>
<tr>
<td>5.</td>
<td>Sepharose 4B (29)*</td>
<td>2.0</td>
<td>0.0040</td>
<td>0.004</td>
<td>0.005</td>
<td>2</td>
<td>0.84</td>
<td>3895.56</td>
</tr>
</tbody>
</table>

*Values in parentheses refers to elution fraction collected number containing PO

$$y = 0.0742x + 0.0613$$

$$R^2 = 0.8932$$
high substrate concentration. Similar, inhibitory effect were reported in PO of *Bombax mori*, by Asano and Ashida [7]. Asano and Ashida [7] also reported a $K_m$ value of 1.9mM for PO of *Bombax mori* by Lineweaver-Burk plot.

Thus from the present study it was concluded that the PO of freshwater crab, *B. cunicularis* has a $K_m$ of 1.21mM and Vmax of 16.31 x10^{-4}.

The PO purification procedure from HLS is shown in Table 1. A highest purity of PO was found in the fraction eluted in Sepharose 4B. The $K_m$ value was determined as 1.2mM (Fig. 1).

The phenoloxidase showed a maximum purification when purified on column chromatography using Sepahrose 4B with an highest specific activity 2. The purified PO on Sepharose 4B was examined for their ability to oxidize L-DOPA at various concentrations. $K_m$ value of the PO for L-DOPA were determined to be 1mM by plotting Lineweaver - Burk plot. A inhibitory effect on enzyme at high concentration was observed. Asano and Ashida [7] reported the $K_m$ value of PO in *Bombax mori* as 1.9 mM using Lineweaver -Burk plot. They too had reported a inhibitory effects on the enzymes at high concentrations. Similar, observation was found in the present study.

Thus, from the present study it was concluded that the PO of freshwater crab, has a $K_m$ of 1.21mM and Vmax of 16.31 x10^{-4}.

**ACKNOWLEDGEMENT**

ERM thank Department of Science and Technology, Government of India for financial assistance.

**REFERENCES**