

Characterization of phenoloxidase of *Penaeus indicus*

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ABSTRACT

The enzyme phenoloxidase from haemolymph of *Penaeus indicus* was partially purified using ammonium sulphate fractionation technique. An overall recovery of 52% with a 5-fold purification was achieved. Dialysis reduced the enzyme activity indicating the presence of dialysable cofactors. The enzyme had high affinity for the biphenolic substrate adrenalin followed by dopamine, catechol and dopa. The rate of the reaction was linear up to 3 min when adrenalin was used as the substrate. The enzyme had an optimum temperature of 50°C but lost 60% of its activity when heated to 70°C for 10 min. The K_m and V_{max} of the enzyme with adrenalin as the substrate was 0.122 mM and 0.588 OD units/mg protein a min respectively. The enzyme was inhibited by the substrate adrenalin at a concentration of 15 mM and by cupric salts. Magnesium and calcium stimulated the enzyme activity. EDTA and mercaptoethanol strongly inhibited the enzyme.

Quinone-tanned proteins or sclerotins are important structural proteins of invertebrate exoskeletons. The role of the enzyme phenol oxidase in sclerotin formation was explained by Pryor (1940 a b) and since then it has been widely reported in parasites (Mansour 1958, Nelliappan *et al.* 1989 a, b), insects (Hackman and Goldberg 1967, Yamazaki 1969, Pau *et al.* 1971, Brunet 1980) and molluscs (Waite and Wilbur 1976, Bharati and Ramalingam 1983). In Crustaceans it was studied mainly in crabs (Summers 1967, Vacca and Fingerman 1975, Nelliappan *et al.* 1982). A few studies were also made in prawns (Antony and Nair 1968, 1975, Nelliappan *et al.* 1989 a, Smith and Soder Hall 1991). Reports that are available pertaining to purification and characterization were made mainly in insects (Ashida 1971, Dohke 1973 Thangaraj *et al.* 1990) and molluscs (Waite and Wilbur 1976, Bharati and Ramalingam 1983).

The present study deals with the partial

purification and characterization of the enzyme phenol oxidase from the haemolymph of *P. indicus*.

MATERIALS AND METHODS

The haemolymph was extracted and collected by piercing the heart by a syringe. The anticoagulant used was 5% trisodium citrate. The collected haemolymph was brought to the laboratory in chilled condition for enzyme and protein assay.

Enzyme assay : Phenol oxidase activity with adrenalin as the substrate was determined according to Preston and Taylor (1970) with minor modifications. The reaction was carried out in 3.0 ml with a final concentration of adrenalin as 0.33 mM in phosphate buffer (pH 7.5, 0.1 M). The enzyme was activated by sodium dodecyl sulphate at the concentration of mg/ml. The formation of adrenochrome was measured spectrophotometrically at 420 nm for 3 min. A unit of enzyme activity was expressed as the amount of enzyme required to form 1 OD unit of adrenochrome in 3 min. under specified conditions.

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Table 1. Partial purification of phenol oxidase of *P. indicus*

Fraction	Volume (ml)	Total units (OD)	Total protein (mg)	Specific activity	Yield %	Fold purification	Yield (%)	Fold purification
Haemolymph	50	201.00	3514.10	0.0190	100.00	1.00		
0 - 20 %	5.42	39.46	132.51	0.0992	19.63	5.22)		
)	51.77	4.92
20 - 40 %	4.85	64.60	238.620	0.090	32.14	4.74)		
)		
50%	40.56	77.98	1994.41	0.013	38.80	0.684		

Protein assay : Protein was estimated according to Lowry *et al.* (1951).

Partial purification of phenoloxidase enzyme

Haemolymph (50 ml) was collected from prawns at the harvesting fields. Crystalline ammonium sulphate was added with constant stirring to give a 20% saturation. The solution was allowed to stand for about 2 hr, and the precipitated proteins were removed by centrifugation at 10 000 RPM for 15 min at 4°C.

The precipitate was dissolved in minimum quantity of phosphate buffer (pH 7.5, 0.1 M) and dialysed against the same buffer for 3 hr.

The procedure was repeated with the supernatant at ammonium sulphate concentration of 20-40% and 50%. The precipitated proteins at these 3 steps were analysed for enzyme activity and protein concentration.

RESULTS

Partial purification of phenoloxidase enzyme

Phenol oxidase was partially purified by a procedure involving ammonium sulphate fractionation. Almost 5-fold purification with an overall recovery of 52% was achieved (Table 1). Overnight dialysis resulted in re-

duction in enzyme activity indicating the presence of some dialysable co-factors.

Properties of phenoloxidase

The properties of phenol oxidase enzyme was studied by using the 0-20% and 20-40% fractions obtained in the partial purification step.

The rate of adrenochrome formation was linear up to 3 min. Optimum temperature of incubation of phenol oxidase was 50°C. Above this temperature rapid decline in enzyme activity was observed. The results on the effects of heat treatment on the activity of phenol oxidase showed that enzyme was stable up to 45°C. It lost 60% of its activity on heating at 70°C for 10 min. The optimum

Table 2. Oxidation of various substrates by phenoloxidase of *P. indicus*

Substrate	Relative activity	
	at 420 nm	at 475 nm
Epinephrine (control)	100	100
Dopamine	46	52
Dopa	27	29
Catechol	32	18
Tyrosine	00	00
Phenol	00	00

pH of phenol oxidase was recorded at 8.5. A slight decrease in activity was observed from pH 5.0 till 6.5 indicating the possibility of a minor peak below pH 5.0. Substrate specificity of the enzyme was examined with various substrates differing in their side chain as well as their phenolic group. The enzyme showed varying degrees of activity towards the biphenolic substrates differing in their side chain with the order of preference as follows : Adrenalin > dopamine > catechol > dopa. Monophenolase activity was totally absent (Table 2). Lineweaver and Burk plot of the enzyme with adrenalin as the substrate revealed the K_m of the enzyme to be 0.121 mM, and V_{max} for the same was 0.588 OD units/mg protein/min (Table 3). The enzyme was inhibited to some extent by the substrate adrenalin itself at a concentration of 15 mM onwards.

Various metal ions were examined to study their effect on the activity of phenol oxidase (Table 4). Salts of magnesium, manganese and calcium stimulated enzyme activity. Cupric salts at both concentrations and iron salt at the high concentration were inhibitory. The results on the effects of various group-specific agents on the activity of phenol oxidase are presented in Table 5. So-

Table 3. Effect of substrate concentration

Substrate concentration (mM)	1/S	V	1/V
2	0.5	0.509	1.96
1	1	0.494	2.02
0.5	2	0.490	2.02
0.33	3.0	0.415	2.41
0.25	4.0	0.448	2.23
0.167	5.99	0.369	2.71
0.083	12.05	0.237	4.22
0.0416	24.04	0.151	6.62
0.0208	48.08	0.074	13.51

Table 4. Effect of metal ions on phenol oxidase activity

Cations	Relative activity	
	1×10^{-3} M	1×10^{-4} M
Control	100	100
NaCl	97	100
KCl	95	98
MgSO ₄	112	106
MnCl ₂	113	106
CaCl ₂	107	101
CuSO ₄	87	95
CuCl ₂	74	88
CuCl	99	92
FeSO ₄	53	104
ZnSO ₄	98	99
CoSO ₄	102	99

dium metabisulphite and mercaptoethanol strongly inhibited the enzyme. Inhibition of activity was also observed with 1-10 phenanthroline, EDTA and 8-hydroxy quinoline. No inhibition of activity was observed with cysteine-HCl.

DISCUSSION

Purification of the enzyme phenol oxidase has been the subject of study of many workers. The enzyme had been purified from the haemolymph of *Bombyx mori* (Ashida 1971). A prophenol oxidase enzyme was purified from crayfish haemocytes using ammonium sulphate fractionation technique by Ashida and Soder Hall (1984). Thangaraj *et al.* (1990) reported 76% recovery of the enzyme from the haemocytes of the pest *Oryctes rhinoceros* using ultra gel ACA 34. In this study a 5-fold purification with an overall recovery of 52% was observed. However Waite and Wilbur (1976) observed a 5-fold purification with a yield of 31% after using the technique of gel filtration through sephadex G-100.

Table 5. Effect of activators and inhibitors on phenol oxidase activity

Reagent	Relative activity	
	1×10^{-3} M	1×10^{-4} M
Control	100	100
Cysteine - HCl	101	93
8-Hydroxy quinoline	55	94
Sodium metabisulphite	00	00
EDTA	49	41
1, 10-phenanthroline	41	67
Mercaptoethanol	00	00

The properties of phenol oxidase from different sources vary considerably. Mansour (1958) reported the reaction rate to be linear up to 20 min for the enzyme from *Fasciola*. The enzyme from mouse melanoma oxidized adrenalin at a higher rate, and dopa at a lower rate. The reaction was linear for approximately 3 min as the enzyme clearly preferred adrenalin as the substrate. It did not oxidise tyrosine and phenol indicating the absence of monophenolase activity. This is in confirmation with the observations in crab *Emerita asiatica* Milne Edwards (Nellaiappan *et al.* 1982). The enzyme had optimum pH at 8.5. This is contrary to the observations made by Antony and Nair (1968) that in *M. monoceros* phenoloxidase had a very wide pH range. But Waite and Wilbur (1976) reported the enzyme in bivalve *Modiolus demissus* to have a pH range of 8.0-8.5. The optimum temperature of the enzyme was 50°C. This was in agreement with the reports of Antony and Nair (1975) in *M. affinis*. Mansour (1958) reported that enzyme from liverfluke lost its activity completely when boiled for 1 min. In this study the enzyme lost 60% of its activity on exposure to heat at 70°C for 10 min. The phenoloxidase enzyme from coconut pest *Oryctes rhinoceros*

was stable up to 70°C and lost 70-85% of its activity when the enzyme was exposed at 70°C for 10 min (Thangaraj *et al.* 1990).

The K_m and V_{max} of the enzyme using adrenalin as the substrate were 0.121 mM and 0.588 OD units/mg protein/min respectively. The literature with regard to the kinetics of the enzyme in prawn is very scarce. However, Waite and Wilbur (1976) observed a low K_m and high V_{max} for substrates such as 4 methyl-catechol and 4-butyl catechol in the bivalve *Modiolus demissus*.

A substrate-mediated inhibition was also observed by us which shows some control over the enzyme action.

Mansour (1958) and Nellaiappan *et al.* (1989b) reported activation of the enzyme by copper salts in *Fasciola gigantica*. However, cupric salts inhibited the enzyme in this study. The enzyme was severely inhibited by mercaptoethanol indicating the presence of disulphide bridge for its activity. Waite and Wilbur (1976) also observed the requirement of disulphide bridge for the enzyme activity in *Modiolus demissus* Dillwyn. The enzyme was also inhibited by metal chelators such as ethylene diamine tetra acetic acid and 1, 10 phenanthroline. This shows the requirement of certain metals as cofactors by the enzyme for its activity. This is in conjunction with the observation of Waite and Wilbur (1976) and Nellaiappan *et al.* (1989 b). Antony and Nair (1975) reported the inhibitory effect of *L. cysteine* on the enzyme activity in the prawn *M. monoceros*. No such inhibition was observed in the present study.

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