STUDIES ON THE ENZYME PHENOL OXIDASE IN *PENAEUS INDICUS* H. MILNE EDWARDS

DISSERTATION SUBMITTED BY

ANILKUMAR P.

IN PARTIAL FULFILMENT FOR THE DEGREE OF
MASTER OF SCIENCE (MARICULTURE)
OF THE
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

POST GRADUATE PROGRAMME IN MARICULTURE
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
COCHIN-682 031

DECEMBER 1992

C E R T I F I C A T E

This is to certify that this Dissertation is a bonafide record of work carried out by Sri. **Anil Kumar P.** under my supervision and that no part thereof has been presented before for any other degree.

Sridhar N.

Dr. N. SRIDHAR
SCIENTIST
CENTRAL MARINE FISHERIES
RESEARCH INSTITUTE
COCHIN

Countersigned by:

Dr.P.S.B.R.JAMES
DIRECTOR
CENTRAL MARINE FISHERIES
RESEARCH INSTITUTE
COCHIN

CONTENTS

*** *** ***

PREFACE

Modern aquaculture is a new science and industry although traditional in origin. In the past fifteen years aquaculture production shows a steady increase from 5 million metric tonnes (1973) to 14.5 million metric tonnes (1988). In future an increase in the population coupled with an increase in percapita consumption will definitely culminate in the intensified effort to increase aquaculture production. In this context prawns are very important as a candidate species for culture and foreign exchange earner for a developing country like India. In order to achieve a reliable mechanism for production in future, a thorough understanding of the cultured animal is necessary. In prawns the most important event occurring through out its life cycle is moulting, which is a mechanism for growth. Prawns like most other crustaceans are ensheathed in a thick outer covering called exoskeleton. The presence of this rigid exoskeleton limits the growth of this animal, which is solved by the periodic shedding of the exoskeleton followed by the secretion of new ones.

The exoskeleton of prawn is a rigid calcareous structure. Its basic structure consists of a thin outer most layer of lipo-proteinous epicuticle followed by a procuticle. A single layer of epidermal cells lie beneath the procuticle. The epicuticle is further divided into an outer thin layer and an inner tanned, thick layer. The procuticle has a preecdysal and post ecdysal layer, secreted before and after ecdysis, respectively. The

preecdysal layer which is the outer tanned layer of procuticle is also called the exocuticle. The postecdysal layer which is known as endocuticle, consists of an outer calcified principal layer and a thick uncalcified membraneous layer. Extensions of the epidermal cells vertically pass through the pore canals of the cuticle. A basement layer lies under the epidermis. Obviously the epidermis is responsible for the secretion of epicuticle, procuticle and the moulting fluid.

The process of moulting can be divided into three stages namely premoult, intermoult and postmoult. During premoult, the epidermal cells, activated by hormones enlarge in size and start synthesis of cuticular components. At the same time separation of the exoskeleton from the body occurs by the dissolution of the membraneous layer of the old exoskeleton. The epidermis then secretes epicuticle and exocuticle and shrinks to normal size by the time of ecdysis. After ecdysis the epidermis synthesizes endocuticle, which is completed by the secretion of the membraneous layer. In the following intermoult stage the animal prepares for the next moult. The newly moulted animal with a flexible exoskeleton increases in size by water in take. This is followed by hardening of the cuticle.

The exoskeleton of a newly moulted prawn will be soft and pale in colour, but becomes harder over a period of some hours. This hardening process starting from the inner epicuticle and proceeding inwards to the exocuticle is called sclerotization. In the sclerotization process, quinones of the tyrosine metabolism react with the free amino groups of the

structural proteins of the cuticle called Arthroidin, to give structures cross-linked with covalent bonds. The quinones are formed from the o-dihydric phenols by the action of o-dihydroxy phenol oxidase. It has also been found that in the tyrosine metabolism conversion of tyrosine to the final substrate of the enzyme phenol oxidase is triggered by the moulting hormone ecdysone. It also removes the inhibition of the phenol oxidase enzyme. So it can be concluded that hardening of the new cuticle is initiated by phenol oxidase enzyme which in turn is influenced by ecdysone.

It is difficult for the newly moulted prawns to survive with the soft exoskeleton unless the hardening takes place immediately after the swelling of the body by water intake. This may be one of the reasons for the high death rate during moulting. In this context the "soft prawn syndrome" in the prawns deserves special mention. In large numbers P. indicus is found to be affected by it in the culture system. In this disease, the softening of the exoskeleton followed by secondary pathogenic infection results in high mortality.

Apart from moulting, reproduction is another biologically important event in the life history of prawns. In prawns which come under subclass Brachyura reproduction and growth are programmed as antagonistic events. The inter moult period is long enough to accomodate one or more reproductive cycles. In most vertebrates, the ovary plays a dual role in that

it produces egg and also secretes certain hormones. Evidence is accumulating that crustacean ovary also might play a role as an endocrine gland. Hence an attempt has been made to understand the enzyme phenol oxidase by studying its characterization, the enzyme expression in the case of soft prawns and the effect of moulting and some of the reproductive hormones on its biosynthesis.

I am deeply indebted to my supervising guide, Dr. N. Sridhar, for his valuable guidance advise, constant encouragement and patience without which this work would have been incomplete. I am thankful to Dr P.S.B.R. James, director, C.M.F.R.I. for providing the necessary facilities for this work. I am grateful to Mr. Srinath (Scientist) for his helping in statistics. I also thank Dr. A.D.Diwan and Dr. V.K.Pillai for their timely help. I am grateful to Dr.(Mrs) Manpal Kaur Sanhotra for her encouragement at various stages of this work. I thank Shri.A.Nandakumar and Shri. Aboobacker for their help. I am deeply indebted to my classmates especially Shri. C. Ragunathan for his valuable assistance and help. I also wish to thank all my juniors and seniors for their assistance. I express my gratitude to the Indian Council of Agricultural Research for awarding me the Junior Research Fellowship during the tenure of which this work has been carried out.

1. INTRODUCTION

1.1. Occurrence and Characterisation of Phenol Oxidase Enzyme:

O-diphenol oxidase (E.C.1.10.3.1) is interchangeably known as catechol oxidase, tyrosinase, catecholase, polyphenol oxidase, phenol oxidase and phenolase. Non-arthropod catechol oxidases associated with quinone-tanning have been partially purified and characterised mainly from marine mussels Mytilus edulis and Geukensia demissa where they occur in both the byssus and periostracum (Waite and Wilbur, 1976; Waite, 1985).

Smyth (1954) has histochemically demonstrated phenol oxidase in the egg shell formation in helminthes and byssus formation in mytillus. Phenol oxidase from the liver fluke Fasciola hepatica and the effect of serotonin on it has been reported by Mansour (1958). Presence of phenolase in the metacercarial cyst of Fasciola has been reported by Campbell (1960). Phenol oxidase in schistosomes has been reported by Seed et al., (1978) and Seed and Bennet (1980). Nellaiappan et al., (1989) have studied the properties of phenol oxidase in Fasciola gigantica. The phenol oxidase of Fasciola was found to catalyse the oxidation of different phenol derivatives, such as tyramine (4-hydroxyphenyl ethyl amine), 3-hydroxy tyramine, l-epinephrine, tyrosine and catechol. The liver fluke enzyme was found to oxidise l-epinephrine at a much higher rate than the phenol oxidase from Harding-passey mouse melanoma. Boiling the enzyme

preparation for 1 min caused inactivation of the enzyme. Dialysis of the enzyme preparation overnight did not reduce enzyme activity, indicating the absence of any dialysable cofactors. It was found that reaction mixtures were red after incubation with epinephrine and black after incubation with tyramine, hydroxy tyramine and DOPA indicating adrenochrome for epinephrine and probably melanin for other compounds (Mansour, 1958).

In molluscs phenol oxidase is reported in the periostracum of Modiolus demissus (Waite et al., 1976), in the foot of Perna virdis (Bharathi et al., 1983), in the mantle of the snail, Ashatina ulica (Nellaiappan et al., 1989) and in mussels (Rzepecki et al., 1991). Phenol oxidase extracted from Modiolus demissus can be chymotrypsically activated. Molecular weights were 80,000 and 70,000 daltons for the proenzyme and active enzyme respectively. Optimal pH was of 8.0 - 8.5 and adding copper chelators and reducing sulphide bonds was found to be diminishing the enzyme activity. In the former case, activity was restored by titrating the inhibitor with divalent copper. It had a low Km (Waite et al., 1976). Samata et al., (1980) have reported a copper containing catechol oxidase in the organic matrix of all the molluscan shells. This enzyme is water soluble in Musca (Hackman and Goldberg, 1967). An impure version of this enzyme was isolated from Calliphora erthyrocephla (Karlson and Libran, 1961).

In insects, role of phenol oxidase in the formation of ootheca in cockroach was reported by Brunet and Kent (1955), Kent and Brunet (1959), Pau and Acheson (1968), Pryor (1940a,b), Stay and Roth (1962).

The O-diphenol oxidase of fly larvae was reported by Hackman and Goldberg (1967). Yamazaki (1969) observed an insoluble phenol oxidase in the cuticle of Drosophila virilis, Bombyx mori and Pabilio xuthus. The enzyme from cockroach blood was prepared in an active form by Mills et al., (1968). Other reports on insect phenol oxidase are Preston et al., (1970), Pau (1975), Vincent et al., (1979), Brunet (1980) and Kalyani et al, (1985). The larval haemolymph of Lucilia cuprina contains a prophenol oxidase which, when activated by cuticular extract, oxidises a wide range of phenols but is most active on dopamine and 4-methyl alcohol (Hackman and Goldberg, 1967).

A range of marine invertebrates were screened for phenoloxidase in the coelomic fluid or haemolymph. The crustaceans and the ascidian, Ciona intestinalis displayed the strongest activity. The enzyme was found to be present in most crustaceans (Smith and Seederhaell, 1991). Phenol oxidases occur in the blood of a number of crustaceans i.e., Cancer pagurus Homarus vulgaris, Maia squinado and Uca pugnax (Bhagvat and Richter, 1938; Pinhey, 1930 and Sumners, 1967). In Uca and Cancer pagurus the changes in phenol oxidase activity are correlated with changes in epidermal metabolism (Decleir and Vercauteren 1965, 1967). Other reports on phenol oxidase in crustaceans are by Sumners (1967), Vacca and Fingerman (1975), Nellaiappan et al. (1982, 1989), Thangaraj et al. (1987).

The distribution and location of regions with maximum activity of Phenol oxidase was studied in different species of prawns by Antony and Nair (1968). The head juice and tail extracts were found to have

very high order of enzyme activity when compared with cuticle and muscle. Penaeus indicus, Metapenaeus affinis and Metapenaeus monoceros recorded comparatively high enzyme activity than Parapenaeopsis stylifera and Metapenaeus dobsoni. Exposure to higher temperatures up to 55°C was shown to activate the enzyme. Effect of certain chemical agents on phenol oxidase was examined by Antony and Nair (1975). Phenol oxidases from pink and white shrimp was characterized and molecular weight determined by Simpson et al. (1988). The prophenol oxidase activating system in ray fish has been studied by Masaaki Ashida and Kenneth Soderhall (1984).

1.2. Sclerotization

The three requirements for sclerotin formation are a tannable protein, an oxidase system and an ortho-diphenol which requires oxidation to O-quinone before reacting with the protein to harden and darken the exoskeleton (Pryor, 1940 a and b). Locke and Krishnan (1971) observed the oxidation of polyphenols that are secreted into presumptive exo and epicuticle to highly reactive quinones by phenol oxidase. The quinones then tan the chitin-protein complex of the exocuticle and lipo-protein of the epicuticle. The diphenol or tanning agent has several forms. 3, 4-dihydroxy benzoic acid (Prayor et al., 1940a) and 3, 4-Dihydroxy benzyl alcohol (Pau and Acheson, 1968) in the cockroch egg capsule. The cockroch colleterial glands, which are responsible for secretion of the egg capsule, contain N-acetyl nonadrenalin in relatively small amounts (Brunet, 1965). Di-and tri-tyrosine (Anderson, 1970) and catechol (Malek, 1961) have also

been implicated as tanning agents. Recent studies indicate that diphenols with a betahydroxyl group on the side chain may be important in protein tanning (Anderson, 1970; Anderson and Bareet, 1971.

The quinone-tanning or sclerotization is well distributed throughout the animal kingdom. The earliest phylogenitic incidence on phenolase is suggested in parasitic protozoans during sporogenesis in host cells (Monne and Hoing, 1954). In cnidaria, the whip corals (Gorgonacea) (Tidball, 1982) and in solitory hydroids e.g., Pachycerianthus fibriatus and Laomedea flexuosa (Knight, 1970), in all classes of platyhelminthes (Bunke, 1972 and 1979; Ishida and Teshiroji, 1986 and Smyth and Clegy, 1959) and in trematode metacercarial cyst (Dixon, 1965 and Campbell, 1960) quinnone tanning has been reported. In nematoda there is one known case of tanning in the cyst of the potato eelworm Globodera rostrochiensis (Awan and Hominick, 1982). Mollusca exploit quinone-tanning for byssal threads in which both the adhesive pad and thread varnish are tanned (Waite, 1983a). The periostraca is a protenaceous cuticular layer covering the outer surface and mollusc shells (Degens et al., 1967 and Waite, 1983b). Within mollusc bivalve shells, the insoluble shell matrix serves as a kind of tanned grouting for mineral deposits (Degens et al., 1967; Gordon and Carrikor, 1980 and Wheeler et al., 1988).

The tanning agents of crustaceans have not been identified. However, Sumners (1967 and 1968) has demonstrated in vitro that a phenol oxidase exists in the plasma and epidermis of the fiddler crab, Uca pugilator,

during the moulting cycle which can hydroxylate monophenols and oxidize o-diphenols. Tysosine can be converted to N-Acetyl dopamine in blood serum and in carapace preparations to which the epidermis remains attached. Marmaras and Fragaulis (1971) have outlined the possible pathway of 3.4-dihydroxy phenyl alanine (DOPA) metabolism in the midgut gland of the decapod crustacean, Upogebia littoralis. In vitro and in vivo metabolism of ^{14}C-DOPA revealed the presence of dopamine, 3-methoxy dopamine and 3, 4-dihydroxy phenyl acetic acid. In another study, Mormaras et al. (1971), again using the midgut gland of U. littoralis, presented the pathway for the metabolism of ^{14}C-Dopamine to noradrenalin, adrenalin, normetanephrine and metanephrine. Vacca and Fingerman, (1975) identified labelled N-acetyldopamine, N-acetyl noradrenalin and their beta-glucoside forms by paper chromatography as metabolites of labelled depamine in the blood of the Fiddler crab, Uca pugilator undergoing ecdysis. They also reported two proteins which act as carriers in the transport of tanning agents into the cuticle.

1.3. Role of steroid hormones in moulting and reproduction

a) Ecdysone

The control of moulting by the hormone ecdysone is well documented (King and Siddall, 1969; Chang et al. 1976a and b; Chang and O'Connor, 1977 and 1978). Galbraith et al. (1969) have suggested that crust ecdysone (= Beta ecdysone and 20 - hydroxy ecdysone) is the main hormone present in the brown fly larvae at the time of puparium formation and the alpha ecdysone forms the precursor for it. Ecdysone induces the formation

of the enzyme DOPA carboxylase needed for the conversion of DOPA to dopamine, which is the substrate for the phenol oxidase enzyme (Mills et al., 1967b). Crust-ecdysone is also associated with both cuticle shedding and cuticle hardening in crustaceans (Faux et al., 16969) and it stimulates moulding in a variety of arthropods (Krishna Kumaran and Schneiderman, 1968 and 1969). Another hormone Bursicone mediates tanning in the cuticle of adult fly and a large number of other insects (Fraenkel and Hsia, 1965 and Mills, 1967). It controls the turnover of tyrosine and tyrosine O-phosphate and, the deposition of the postecdysal endocuticle (Seligman et al. 1969 and Fogal and Fraenkel,1969a). In the cockroach, bursicon initiates the synthesis of dopamine (White head, 1969).

Experiments in which a hormone is injected into animals and its effects mentioned are procedurally simple. Early attempts to determine the role of ecdysteroids on the moult cycle were scored as negative. The parameters monitored in those experiments were exuviation (Adelung, 1967 and Lowe et al., 1968) and regeneration (Skinner and Graham, 1970 and Hopkins et al., 1979). But it has also been reported that following injections of 20-hydroxy ecdysone, eyestalkless cray fish moulted earlier than uninjected animals, where as unoperated animals did not respond (Lowe and Horn, 1967 and Lowe et al. 1968). Siu-Ming chan, et al., (1990) investigated the effect of 20-hydroxy ecdysone injection and eye-stalk ablation on the moulting cycle of the shrimp Penaeus vannamei. They found that the injection of 20 - hydroxy ecdysone into intact inter-

moult animals shortened the duration of the intermoult and the early premoult stages and the 20 - hydroxy injection also increased mortality and abnormal setal development. Death usually occurred at the time of ecdysis.

b) Progestrone and Estrogen:

In crustacea, the role of ovary as an endocrine gland is far from established. Evidence is accumulating suggesting that the crustacean ovary might play a role in the biosynthesis of steroid hormones. Estrogen was detected in ovaries and freshly spawned eggs of American lobster, Homarus americanus by Donahue (1940 and 1948). Subsequently, Teshima and Kanazawa (1971) have showed that ovaries of Portunus trituber culatus possess the enzymes, steroid 17-alpha-hydroxidase, steroid 21-hydroxidase and steroid C_{17}-C_{20} lyase, involved in the conversion of progesterone to 17 alpha-hydroxy progesterone, testosterone, and deoxycortico sterone.

Only a very few reports are available on steroid metabolism in decapod crustaceans (Teshima and Kanazawa, 1971 and 1979; Bomirski and Weck, 1976). Kulkarni et al. (1979) observed that injection of progesterone on alternate days into prawns parapenaeopsis hardwickii caused a significant increase in the ovarian index and oocyte diameter after 10 and 20 days. Nagabhushanam et al., (1982) reported 17-hydroxy progesterone to be inducing spawning in the female prawn Parapenaeopsis stylifera when injected at 20°C at which the prawn naturally does not spawn.

2. MATERIALS AND METHODS

2.1. Chemicals

Dopa, Dopamine, Bovine Serum Albumin, Beta Ecdysone, Progesterone, 2-Mercapto ethanol, Tris-HCL, Sodium Dodecyal Sulphate were procured from Sigma Chemical Company, USA. Adrenalin was procured from S.D. fine chemicals. 17-Beta Estradiol was procured from Astra, Idl. Ltd. Other reagents used were of analytical grade and was supplied by either BDH or Merck.

2.2. Animals:

Animals required for the experimentation were procured from the perennial culture ponds of Edavanakkadu area of vypeen Island, Ernakulam. They were then transported to the laboratory in the live condition and allowed to acclimatize. The prawns were fed with fresh clam meat in the laboratory conditions. From these animals, the early premoult animals were selected and divided into three groups. The first group of premoult animals were used as control while the second and third group were used for experiments with progesterone and 17-Beta estradiol hormones respectively. The remaining animals were allowed to moult and when they entered the intermoult stage, were divided into two groups. One group was used as control and the other group was used for experiments with Beta ecdysone hormone.

Soft prawns, identified by a rigid rostrum and soft body and wavy, enlarged intestine was collected from a perennial pond affected by soft prawn syndrome. Normal prawns collected from the same pond were used as control.

2.2.1. Haemolymph collection:

The haemolymph was collected by piercing the heart with a number twenty six stainless steel needle and is withdrawn into a one ml syringe and stored in a polyethylene vial and syringes were rinsed with 5% tri-sodium citrate solution before use. The collected haemolymph was then transported to the laboratory in the chilled condition for enzyme and protein assay.

2.2.2. Exoskeleton extract:

The exoskeletons from the prawns were hand peeled and separated into five parts namely, Rostrum, Carapace, Abdomen, Telson and Uropod. Each part is then separately homogenized with pestle and mortar using a definite quantity of phosphate buffer (pH 7.0, 0.1M) and then centrifuged. The supernatant is preserved for protein and enzyme assay in a deep freezer. All operations were carried in cold conditions.

2.3. Enzyme assay

Phenol oxidase activity of the haemolymph and exoskeleton extracts,

namely Rostrum, Carapace, Abdomen, Telson and Uropod with adrenalin as the substrate was determined by the procedure of preston and Taylor, (1970) with slight modifications. The reaction was carried out in a volume of 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 Adreno-chrome was measured spectrophotometrically at 420 nm upto a period of 3 minutes.

A unit of enzyme activity was expressed as the amount of enzyme required to form one OD unit of adrenochrome in three minutes under specified conditions.

2.4. Estimation sample protein:

Protein was estimated according to the method of Lowry et al., (1951) using the following reagent.

2.4.1. Folin ciocalteu (F.C.) Reagent:

The ready made reagent from BDH company was used. The reagent was diluted with distilled water in the ratio 1:2 before use.

2.4.2. Alkaline Reagent:

A. 2% Na_2 Co_3 in 0.1 N NaoH

B. 0.5% $CuSo_4$, $5H_2O$ in 1% sodium potassium tartarate.

Fifty ml of solution A were mixed with 1 ml of solution B before use.

2.4.3. Method of protein estimation:

One ml of suitably diluted protein sample was mixed with 5.0 ml of freshly prepared alkaline reagent and kept for 10 min. Then, 0.5 ml of F.C. reagent was added to it, shaken vigorously and allowed to stand for 30 min. for development of colour. The intensity of colour was measured spectrophotometrically at 660 nm and the protein concentration in the sample was extrapolated using the standard curve prepared by using bovine serum albumin.

2.5. Partial purification of phenol oxidase enzyme:

About 50 ml of haemolymph was collected from prawns as mentioned earlier. Crystalline ammonium sulphate was added with constant stirring to give a 20% saturation. The solution was allowed to stand for about 2 hrs and the precipitated proteins were removed by centrifugation at 10,000 g for 15 minutes 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 hrs.

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

2.6. Properties of phenol oxidase:

2.6.1. Effect of time of incubation: The enzyme was incubated with the substrate adrenalin , the reaction is followed at 30 seconds interval for 10

minutes.

2.6.2. Incubation temperature:

Reaction mixture containing the partially purified enzyme and adrenalin was incubated at 20, 28, 30, 35, 37, 40, 45, 50, 55, 60, 65°C for three minutes and assayed for enzyme activity.

2.6.3. Heat treatment:

The enzyme was exposed to different temperatures viz. 28, 40, 45, 50, 55, 60 and 70°C for 3 minutes. The tubes containing the reaction mixture were cooled immediately in an ice-bath. The residual enzyme activity was measured in each case.

2.6.4. pH:

The enzyme was assayed at different pH values ranging from 5.0 - 10.0 with an interval of 0.5 citrate phosphate, phosphate and Tris-Hcl buffer were used between 5.0 - 6.0, 6.5 - 7.5 and 8.0 - 10.0 respectively.

2.6.5. Substrate specificity:

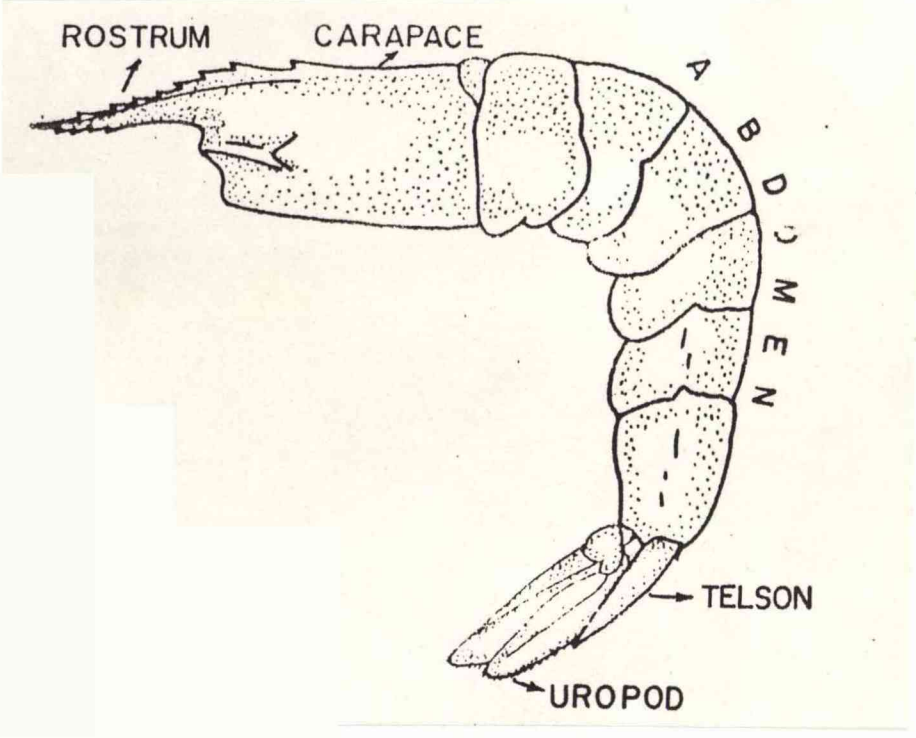
Various substrates namely adrenalin , Dopamine, Dopa, Catechol, tyrosine and phenol at a final concentration of 0.33 millimolar were used to determine the substrate specificity of the enzyme at two different wave lengths of 420 nm and 475 nm.

PLATE - 1

Part of the experimental set up.

PLATE - 2

Various parts of the exoskeleton used For the extraction of the enzyme.


ROSTRUM
CARAPACE
ABDOMEN
TELSON
UROPOD

2.6.6. Substrate concentration:

Using adrenalin as the substrate lineweaver and Burk plots were drawn between the inverse of the substrate concentration and velocity of the reaction to determine the k_m and Vmax of the enzyme.

2.6.7. Effect of metal ions:

The enzyme was incubated with various metal ions in concentrations of 10^{-3} M and 10^{-4} M and then assayed for enzyme activity.

2.6.8. Effect of activators and inhibitors:

Effect of reducing agents, inhibitors and chelating compounds on phenol oxidase activity was studied by incubating the enzyme with the activators and inhibitors to give a final concentration of 10^{-3} M and 10^{-4}M.

2.7. Experimentation:

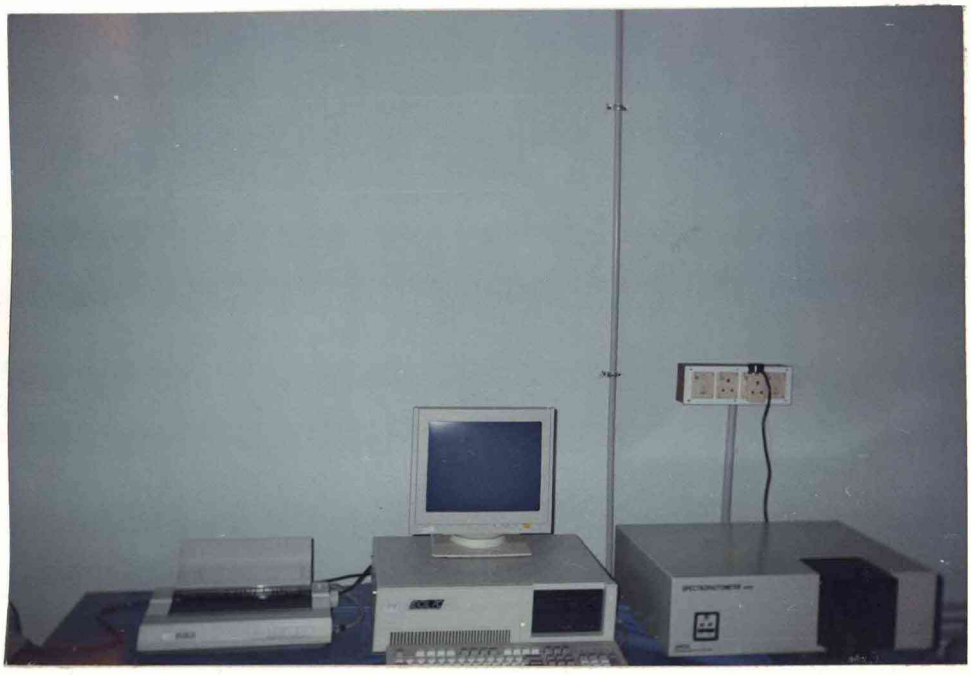
The prawns that were in the premoult stage was separated into three groups as control, progresterone injected and estradiol injected for studying the effect of progestrone and 17-Beta estradiol on phenol oxidase synthesis. The experiment was done in triplicates, with group one as control, groups two and three were given progesterone and 17-Beta estradiol injections respectively, at the rate of one microgram per gram of the body weight after dissolving in acetone. The intermoult prawns were also segregated into two groups, one control and the other experimental.

PLATE - 3.

Spectrophoto meter (ECIL - G55702 used for enzyme assay.

PLATE,- 4

Set up showing dialysis of the enzyme.



The latter group was given ecdysone injections at the rate of one microgram per gram of the body weight. The ecdysone preparation was prepared by dissolving ecdysone in aqueous ethanol. Injections were given at the base of the fifth pedipod, laterally on the muscle on the first and third day of the experiment in the morning and at the end of the third day the animals were sacrificed and the haemolymph collected. Exoskeletal extracts were also prepared as mentioned earlier for use in the enzyme and protein assay.

3. RESULTS

3.1. Partial purification of phenol oxidase enzyme

Phenol oxidase was partially purified by a procedure involving ammonium sulphate fractionation. Almost five fold purification with an overall recovery of 52% was achieved (Table-1). Overnight dialysis was found to result in reduction in enzyme activity indicating the presence of some dialysable co-factors.

3.2. Properties of phenol oxidase of P. indicus

3.2.1. Effect of time of incubation

The rate of adrenochrome formation was observed to be linear up to a period of 3 minutes (Fig.1).

3.2.2. Incubation temperature

Optimum temperature of incubation of phenol oxidase was recorded at 50°C. Above this temperature rapid decline in enzyme activity was observed (Fig.2).

3.2.3. Heat treatment

The results o the effect of heat treatment on the activity of phenol oxidase are presented in Fig. 3. The enzyme was stable upto a temperature of 45°C. It lost 60% of its activity on heating at 70°C for 10 minutes.

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			

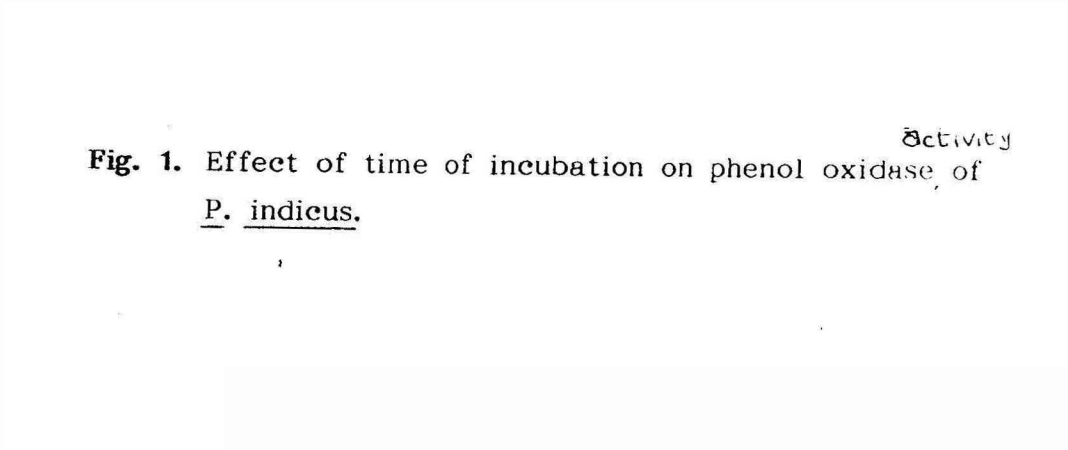


Fig. 1. Effect of time of incubation on phenol oxidase of P. indicus.

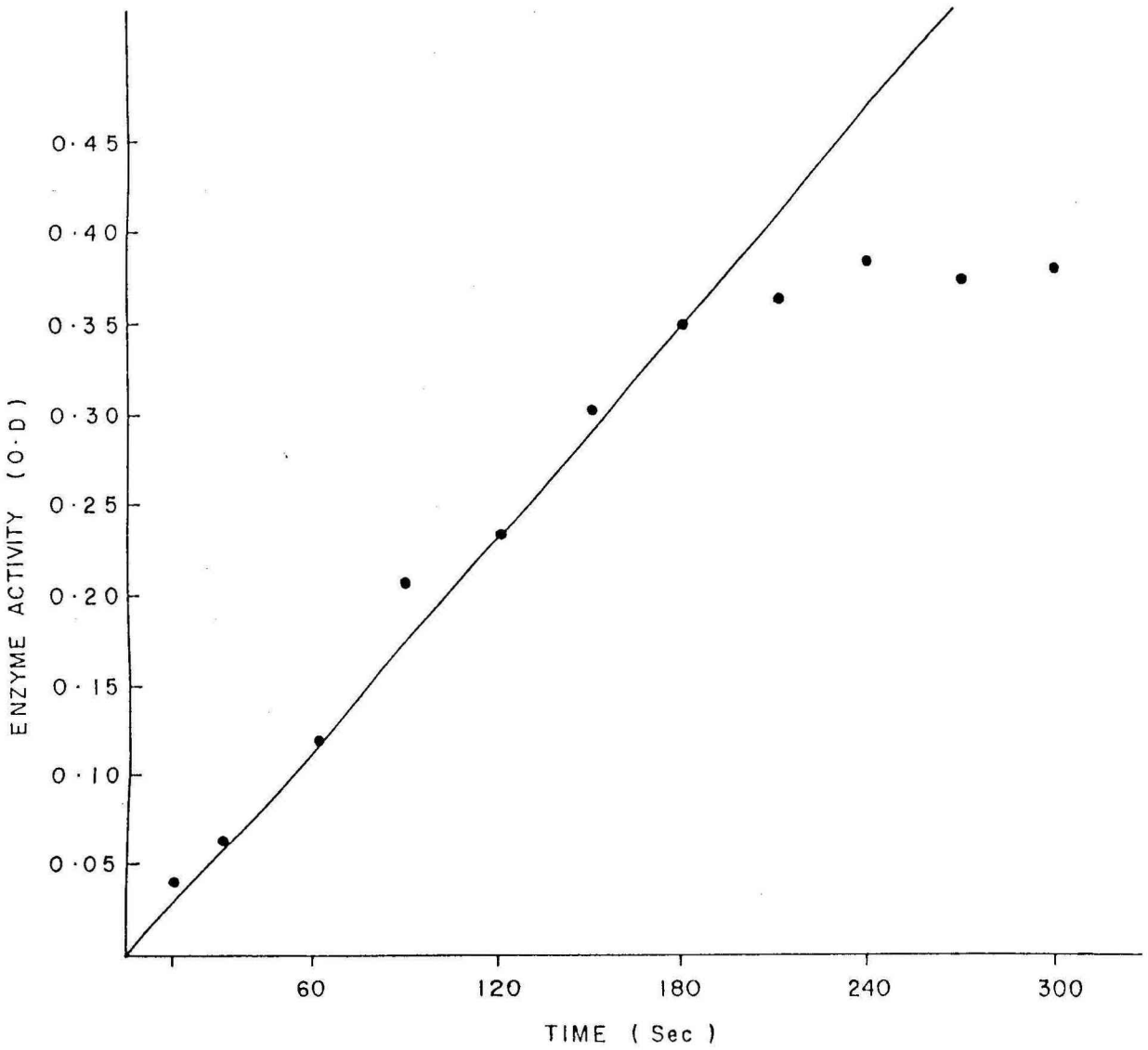

ENZYME ACTIVITY (O·D)
0·45
0·40
0·35
0·30
0·25
0·20
0·15
0·10
0·05
60
120
180
240
300
TIME (Sec)

Fig. 1.

Fig. 2. Incubation temperature of phenol oxidase in P. indicus.

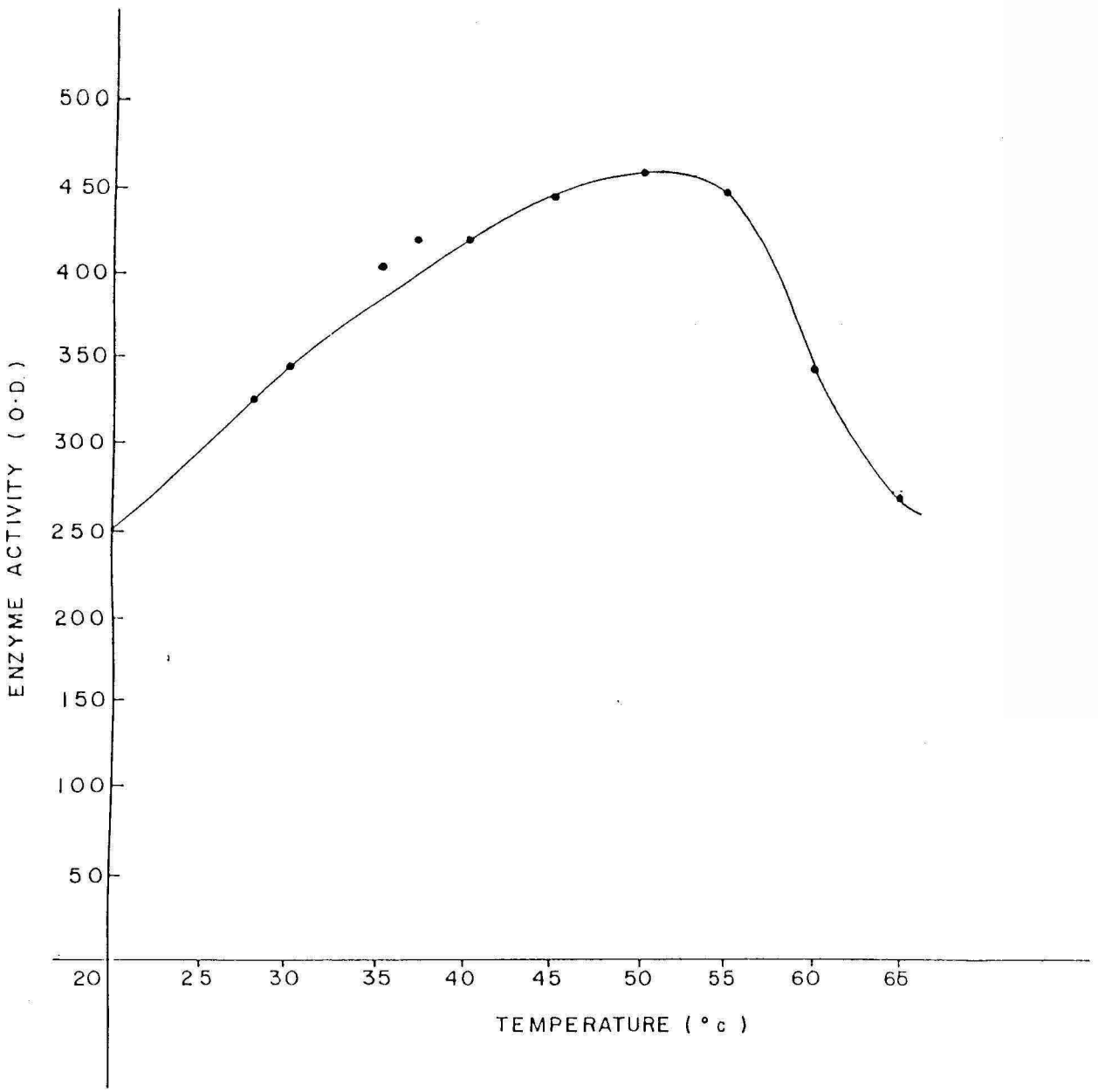

ENZYME ACTIVITY (O·D)
500
450
400
350
300
250
200
150
100
50
20
25
30
35
40
45
50
55
60
65
TEMPERATURE (°c)

Fig. 2.

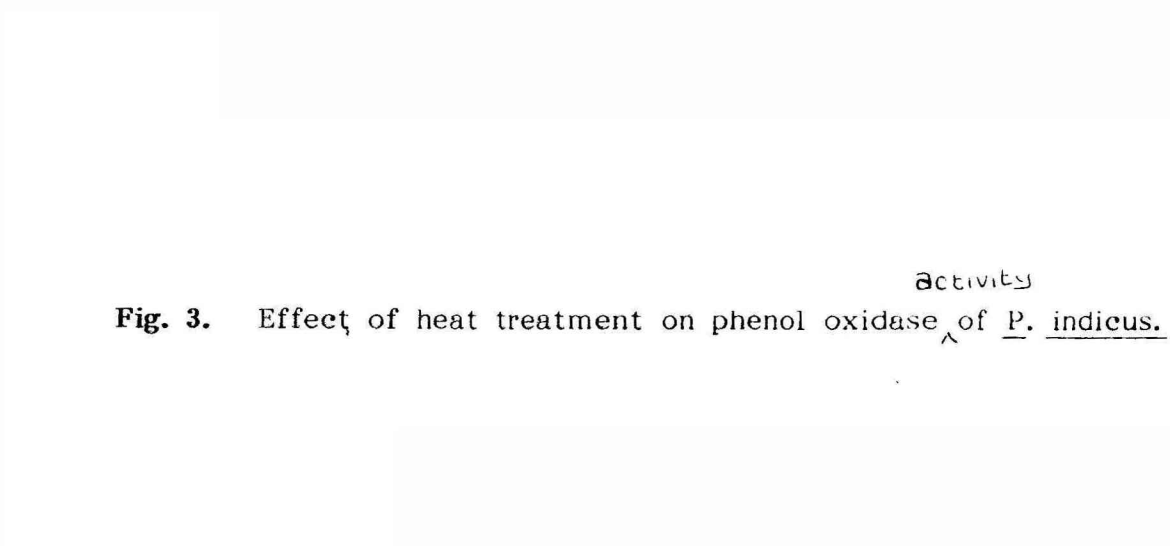


Fig. 3. Effect of heat treatment on phenol oxidase activity of *P. indicus*.

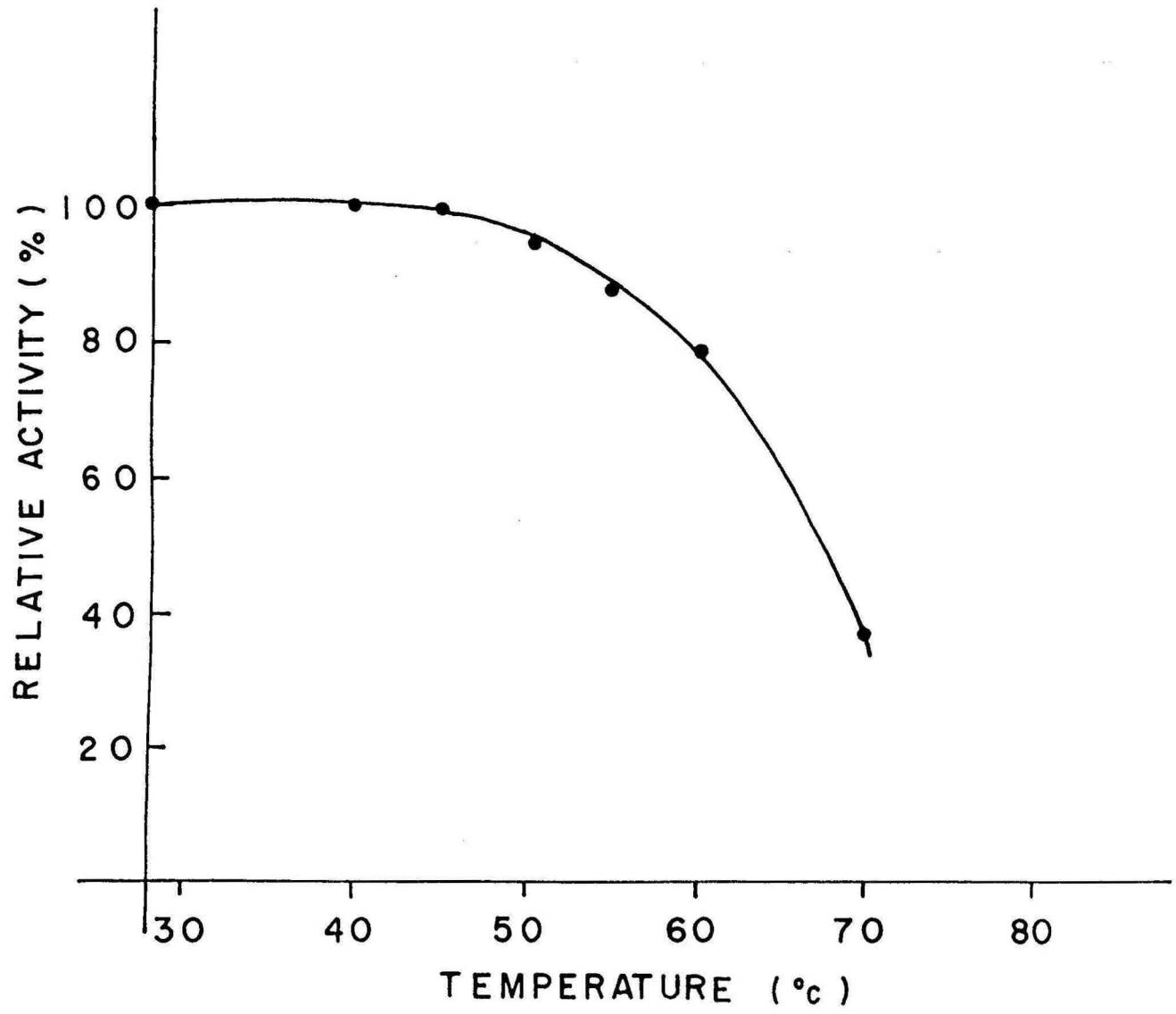

RELATIVE ACTIVITY (%)
100
80
60
40
20
30
40
50
60
70
80
TEMPERATURE (°C)

Fig. 3.

3.2.4. Effect of pH:

The optimum pH of phenol oxidase was recorded at 8.5 (Fig.4). 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.

3.2.5. Substrate specificity

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 bi-phenolic substrates differing in their side chain with the order of preference as follows:- Adrenalin > Dopamine > Catechol > Dopa. Monophenolase activity was totally absent (Table-2).

3.2.6. Substrate concentration

Lineweaver and Burk plot of the enzyme with adrenalin as the substrate has been presented in Fig. 5 and Table 3. The Km of the enzyme was calculated to be 0.129 mM and Vmax for the same was observed to be 0.870 μmoles/mg protein/min.

3.2.7. Effect of Metal ions

Various metal ions were examined to study their effect on the activity of phenol oxidase (Table 4). Salts of magnesium, manganese and calcium were observed to stimulate enzyme activity. Cupric salts at both concentrations and iron salt at the high concentration was found to be inhibitory.

Fig. 4. Effect of pH on phenol oxidase of P. indicus.

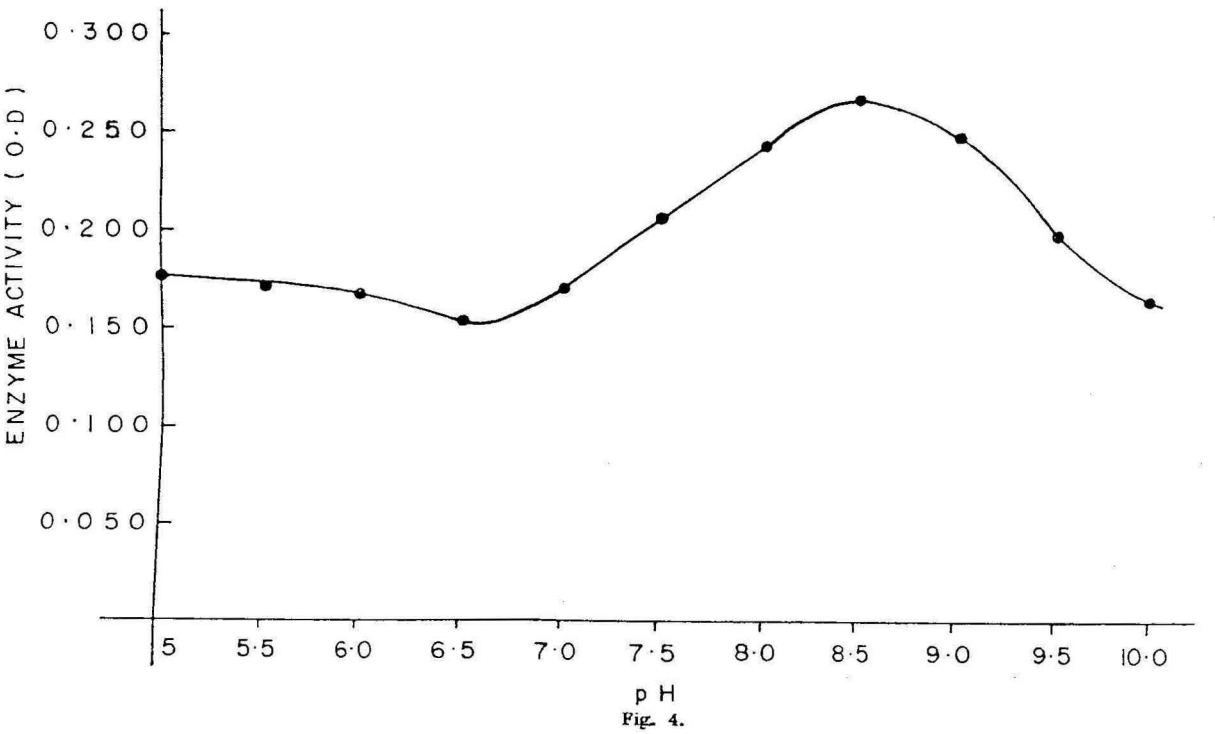

ENZYME ACTIVITY (O·D)
0·300
0·250
0·200
0·150
0·100
0·050
5
5·5
6·0
6·5
7·0
7·5
8·0
8·5
9·0
9·5
10·0
p H

Fig. 4.

TABLE - 2

OXIDATION OF VARIOUS SUBSTRATES BY PHENOL OXIDASE OF P. INDICUS

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

Fig. 5. Line weaver and Burke plot of phenol oxidase in *P. indicus*

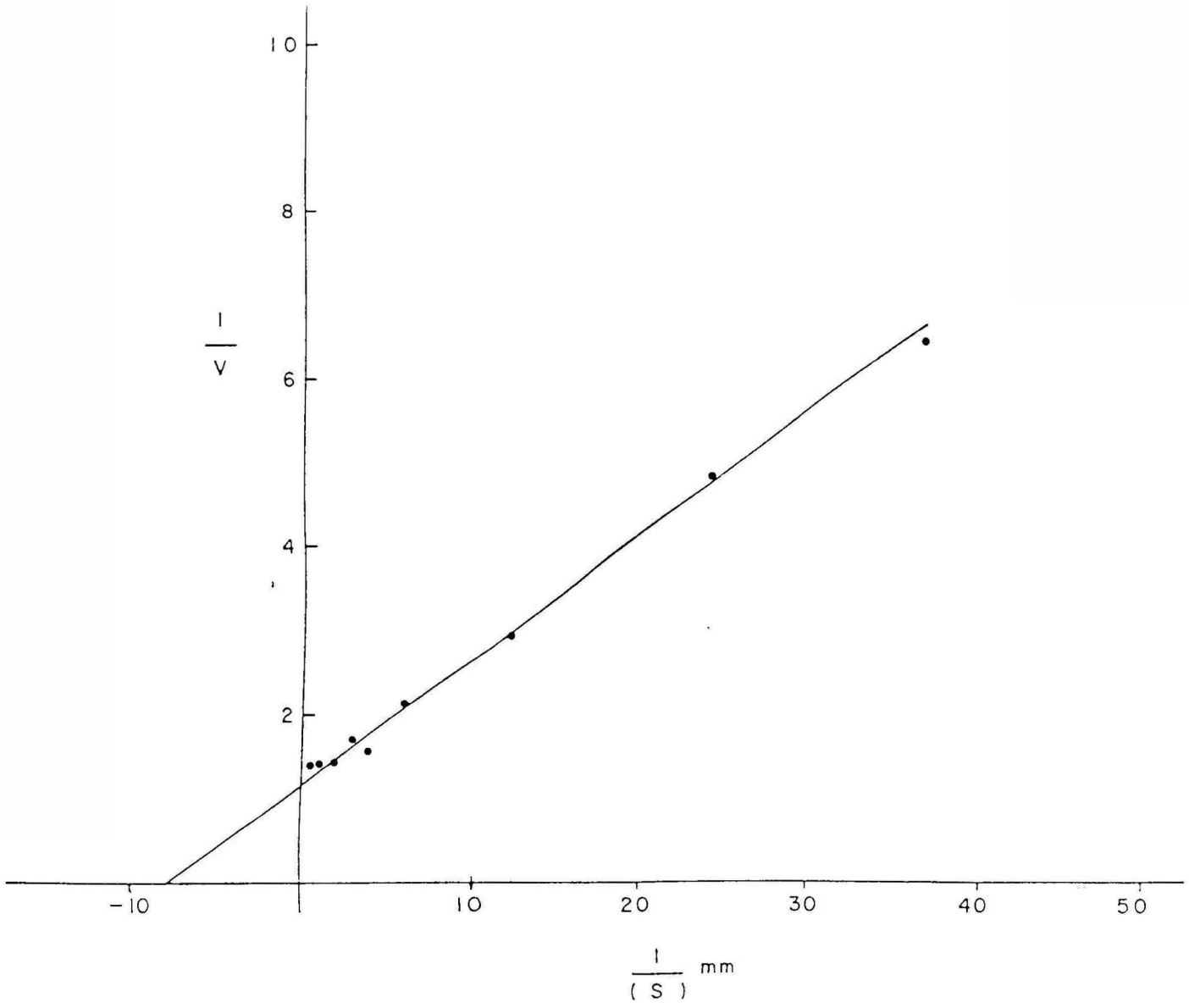


Fig. 5.

TABLE - 3

EFFECT OF SUBSTRATE CONCENTRATION

Substrate concentration (mM)	1/S	V	1/V
2	0.5	0.72	1.40
1	1	0.70	1.43
0.5	2	0.70	1.43
0.33	3.0	0.59	1.69
0.25	4.0	0.63	1.58
0.167	5.99	0.48	2.10
0.083	12.05	0.34	2.97
0.0416	24.04	0.21	4.87
0.0208	48.08	0.099	10.14

T A B L E - 4

EFFECT OF METAL IONS ON PHENOL OXIDASE ACTIVITY

Cations	Relative Activity	
	1 x 10^{-3}M	1 x 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

3.2.8. Effect of activators and inhibitors

The results on the effect of various group specific agent on the activity of phenol oxidase are presented in Table 5. Sodium metabisulphite and Mercaptoethanol very 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.

3.3. Experimentations

The haemolymph and exoskeleton extracts obtained from the animals injected with beta ecdysone as well as the control group (intermoult stage) was assayed for phenol oxidase activity. The results are expressed as specific activity (OD units/mg protein/min.)

3.3.1. Ecdysone injection studies

4.3.1.1. Haemolymph: A highly significant increase in the enzyme activity was observed (0.0505 ± 0.022) in the haemolymph of ecdysone injected animals compared to the intermoult control (0.031336 ± 0.099) group (Table 6).

3.3.1.2. Exoskeleton extracts: A highly significant increase in enzyme activity was observed (Table 8) in the extracts of Rostrum (0.14327 ± 0.08516), carapace (0.16398 ± 0.1461) Telson (0.41324 ± 0.2239), and Uropod (0.05366 ± 0.0346) compared to the control group (Table 7). The

T A B L E - 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
Mercapto Ethanol	00	00

TABLE - 6

EFFECT OF ECDYSONE ON PHENOL OXIDASE ACTIVITY IN THE HAEMOLYMPH OF P. INDICUS

Animals	Specific activity 1	2	3	(Mean) $\bar{X} \neq$	t value
Control (Intermoult)	0.0326	0.0306	0.0307	0.031336 (±0.0099)	3.98**
Ecdysone injected	0.0501	0.0491	0.0522	0.0505 (±0.022)	

TABLE - 7

EFFECT OF ECDYSONE ON PHENOL OXIDASE ACTIVITY IN EXTRACTS OF EXOSKELETON OF P. INDICUS

	Mean specific activity				
	Rostrum	Carapace	Abdomen	Telson	Uropod
Control (Intermoult)	0.0728 (±0.042)	0.09297 (±0.079)	0.08234 (±0.0309)	0.20325 (±0.14886)	0.09492 (±0.07052)
Ecdysone injected	0.14327 (±0.08156)	0.16398 (±0.1461)	0.06505 (±0.0338)	0.41324 (±0.2239)	0.05366 (±0.0346)
't' values	3.759**	2.09**	1.848NS	3.826**	2.57*

** - HIGHLY SIGNIFICANT AT 1% LEVEL
* - SIGNIFICANT AT 5% LEVEL
NS - NOT SIGNIFICANT

The exoskeleton at the abdominal region was found to be detached and hence change in the enzyme ativity was found to be statistically not significant in the abdomen (0.6505 ± 0.0338)

3.3.2. Progestrone injection studies

The haemolymph and the exoskeleton extracts obtained from the animals injected with progesterone as well as the control group (premoult stage) was assayed for phenol oxidase activity. The results are expressed as specific activity (OD Units/mg protein/min.)

3.3.2.1. Haemolymph: No significant change in the enzyme activity was observed (0.04788 ± 0.0448) in the haemolymph of progesterone injected animals compared to the premoult control group (Table 8).

3.3.2.2. Exoskeleton extracts: A highly significant decrease in enzyme activity was observed in extracts of Rostrum (0.06542 ± 0.03861), carapace (0.05292 ± 0.2929), Telson (0.0512 ± 0.0249) and Uropod (0.06516 ± 0.03169) compared to the premoult group exoskeleton (Table 9). The change in enzyme activity in the Abdomen (0.06462 ± 0.01776) was observed to be statistically not significant.

3.3.3. Estradiol injection studies:

The haemolymph and the exoskeleton extracts obtained from the animals injected with estradiol was assayed for phenoloxidase activity. The results are expressed as specific activity (OD units/mg protein/min).

3.3.3.1. Haemolymph: No significant change in the enzyme activity was observed (0.04388 ± 0.0078) in the haemolymph of the estrodiol injected animals compared to the premoult control group (Table 8).

3.3.3.2. Exoskeleton extracts: A highly significant decrease in enzyme activity was observed in extracts of carapace (0.05783 ± 0.03124) Telson (0.07837 ± 0.0439) and Uropod (0.097 ± 0.077) compared to the premoult group exoskeleton (Table 9). The change in enzyme activity in Rostrum and abdomen was observed to be statistically not significant.

3.4. Phenoloxidase activity in soft prawns

Phenol oxidase activity was estimated in the haemolymph and exoskeleton extracts from the soft as well as normal prawns (intermoult stage). The results are expressed as specific activity (OD units/mg protein min.).

3.4.1. Haemolymph:

A highly significant increase in enzyme activity was observed (0.04131 ± 0.0146, 3.81**) in the haemolymph of soft prawns compared to the normal group (0.02323 ± 0.0083).

3.4.2. Exoskeleton extracts:

A highly significant increse in enzyme activity was observed (Table 10) in the extracts of Rostrum (0.055 ± 0.0233; 3.012**), Carapace

TABLE - 8

EFFECT OF PROGESTERONE AND ESTRADIOL ON THE PHENOL OXIDASE ACTIVITY IN THE HAEMOLYMPH OF P. INDICUS

	1	2	3	$\bar{x}$	t value
Control (Premoult)	0.0411	0.0408	0.0428	0.04067 (±0.0078)	1.8437 NS
Progesterone injected	0.0492	0.0475	0.0470	0.04788 (±0.0448)	1.4438 NS
Estradiol injected	0.0438	0.04475	0.0430	0.04388 (±0.0078)	

TABLE - 9

EFFECT OF PROGESTERONE AND ESTRADIOL ON PHENOL OXIDASE ACTIVITY IN THE EXTRACTS OF EXOSKELETON OF P. INDICUS

	Rostrum	Carapace	Abdomen	Telson	Uropod
Control (Premoult)	0.13357 (±0.04255)	0.08114 (±0.0279)	0.0679 (±0.0394)	0.54385 (±0.12769)	0.56675 (±0.1684)
Progesterone injected	0.06542 (±0.03861)	0.05292 (±0.02929)	0.06462 (±0.01776)	0.0512 (±0.0249)	0.06516 (±0.03169)
't' values	5.81**	3.415**	0.3718NS	18.55**	14.34**
Estradiol injected	0.10896 (±0.0543)	0.05783 (±0.03124)	0.05675 (±0.0833)	0.07837 (±0.0439)	0.097 (±0.077)
't' values	1.75NS	2.72**	1.125NS	16.891**	12.42**

(0.021 ± 0.0118, 3.60**), abdomen (0.0276 ± 0.015, 3.98**) and Uropod (0.0112 ± 0.0088; 3.29**) compared to the control group. A significant increase in enzyme activity was observed in the Rostrum of soft prawns when compared with exoskeleton extracts from other regions from the same group (Table 11).

T A B L E - 10

PHENOL OXIDASE ACTIVITY IN THE EXTRACTS OF EXOSKELETON OF SOFT PRAWNS

	Rostrum	Carpace	Abdomen	Telson	Uropod
Control (intermoult)	0.0231 (±0.029)	0.00598 (±0.0067)	0.0069 (±0.0077)	0.01271 (±0.0189)	0.00289 (±0.0027)
Softprawns	0055 (±0.0233)	0.021 (±0.0118)	0.0276 (±0.015)	0.0264 (±0.0194)	0.0112 (±0.0088)
't' values	3.012**	3.60**	3.98**	1.72NS	3.29**

T A B L E - 11

COMPARISON OF PHENOL OXIDASE ACTIVITY BETWEEN ROSTRUM AND OTHER PARTS OF THE EXOSKELETON IN SOFT PRAWNS

	't' value
Rostrum Vs. Carapace	6.172**
Rostrum Vs. Abdomen	4.67**
Rostrum Vs. Telson	3.643**
Rostrum Vs. Uropod	8.477**

4. DISCUSSION

4.1. Partial purification of phenol oxidase of Penaeus indicus

The enzyme o-Diphenol oxidase catalyses the oxidation of o-diphenol to an o-quinone which react with a tannable protein to harden and darken the exoskeleton (Pryor, 1940a,b). The enzyme has been reported in the liverfluke Fasciola hepatica (Mansour, 1958), insects (Hackman and Goldburg, 1967; Yamazaki, 1969; Preston et al., 1970; Burnet, 1980 and Kalyani, et al., 1985), in crustaceans (Sumners, 1967; Vacca and Fingerman, 1975; Nellaiappan et al., 1982, Thangaraj et al. 1987, and Nellaiappan et al., 1989 . The enzyme has been partially purified and characterised mainly from marine mussels. (Waite and Wilbur, 1976; Waite, 1985). The enzyme has been purified by a procedure involving ultrafiltration followed by gel filtration using sephadex G-100 with an over all recovery of 31%. The literature on purification of the enzyme from crustaceans is very scanty. In this study using ammonium sulphate fractionation the enzyme was partially purified from the haemolymph of the prawn P. indicus with a five fold purification and yield of 52%.

4.2. Properties of phenol oxidase

Properties of phenol oxidase from different sources vary considerably. In the case of phenol oxidase from Fasciola the reaction rate was linear for approximately 20 minutes. Catechol and Dopa were oxidised at a lower rate. The enzyme from liver fluke and from mouse melanoma oxidised dopamine at a higher rate. The enzyme from mouse melanoma

also oxidized adrenalin at a higher rate where as dopa was oxidised at a lower rate (Mansour, 1958). In the present study the reaction was linear for approximately 3 minutes. The enzyme clearly preferred adrenalin as the substrate. It did not oxidise tyrosine and phenol indicating the absence of monophenolase activity. This is in conformation of the observations of Nellaiappan et al. (1982). The enzyme had optimum pH at 8.5. This is contrary to the observations made by Antony and Nair (1968) with regard to the phenol oxidase activity in M. monoceros which had a very wide pH range. Waite and Wilbur (1976) reported the enzyme in bivalve Modiolus demissus to have a pH range between 8.0 - 8.5. In the present study the enzyme was found to have an optimum temperature of about 50°C which was in agreement with the study made in M. affinis by Antony and Nair (1975). Mansour (1958) reported the enzyme from liver fluke to have lost its activity completely by boiling for one minute. In the present study the enzyme lost 60% of its activity on exposure to heat at 70°C for 10 minutes. The km and Vmax of the enzyme in the present study using adrenalin as the substrate was 0.129 mM and 0.870 /u moles/mg protein/min. respectively. The literature with regard to the kinetics of the enzyme in prawns is very scarce. However Waite and Wilbur (1976) reported a low Km and high Vmax for substrate with high partition coefficients in 2-octanol : water such as 4-methyl catechol and 4-butyl catechol in bivalve Modiolus demissus Dillwin. The enzyme phenol oxidase in the present study was inhibited by copper sulphate. However an increase in phenol oxidase activity was reported in the liver fluke when copper sulphate was added to the reaction mixture by Mansour

(1958). Waite and Wilbur (1976) observed restoration of enzyme activity to Diethyl dithio carbomate inhibited enzyme on the addition of equimolar amount of cupric chloride. In the present study cupric chloride inhibited the enzyme. The enzyme phenol oxidase was found to be severely inhibited by Mercapto ethanol and sodium metabisulphite. This shows the enzyme to be having a disulphide bridge for its activity. The enzyme was also severely 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). Antony and Nair (1975) reported the inhibitory effect of L-cysteine on phenol oxidase activity of the prawn M. monoceros. No such inhibition was observed in the present study.

4.3. Effect of beta ecdysone, progesterone and estrogen on phenol oxidase activity.

4.3.1. Ecdysone

Butenandt and Karlson (1954) isolated a pure moulting hormone and gave the name ecdysone. About a decade later its chemical structure was elucidated (Huber and Hoppe, 1965). Ecdysone (also called alpha-ecdysone) and Ecdysterone (also called as beta-ecdysone) was first isolated from the pupae of Bombyx mori (Kaplani et al. 1966). Two different moulting hormones referred to as ecdysones have been isolated from crustacea (Nakaneshi et al. 1968). Ecdysterone was extracted from the cray fish Jassus lalandi (Hampshire and Horn 1966; Horn et al. 1968). Injection of ecdysterone induces moulting in isopod Arma dillition bulgari

and in decapods proambrus species and Euca pugillator. Crustacean also react to the insect moulting hormone ecdysone and to phyto-ecdysones (Krishnakumaran and Schneiderman, 1968). Injection of ecdysone into Calliphora erythrocephala larvae, which have been ligated shortly before pupation, induces synthesis of several enzyme particularly of dopadecarboxylase. Since the blockage of DNA-dependent RNA synthesis prevents this effect, the hormone appears to control the transcription of a specific gene coding for the enzyme in question. The RNA isolated from ecdysone treated insects had about six times higher template activity for the dopa decarboxylase than RNA from controls (Karlson, 1966; Karlson and Sekeris, 1966). Exposure of cells or nuclei to ecdysone induces the formation of specific puffs occurring normally in the course of the moulting process (Berendes, 1967). This cytological observation complements the bio-chemical findings that ecdysone stimulates the synthesis of specific messenger RNAs. The hypothesis that ecdysone acts on the level of transcription thus rests on the assumption that induction of enzyme synthesis or occurrence of puffs are the primary effects. In the present study there was a highly significant increase in phenol oxidase activity in the haemolymph of beta ecdysone injected animals when compared with the control. In the exoskeleton the increase in the enzyme activity was highly significant with regard to rostrum, carapace and telson where as the increase was significant with regard to uropod and non-significant with regards to abdomen. At the end of the experiment the exoskeleton of the abdomen was found to be detached from the underlying body tissue. This explains the results obtained in the case of abdomen.

Lachaise and Hoff-man (1977) reported several ecdysteroids in ovaries of the crab Carcinus maenas using radioimmunological assay. In O. gammarella, the presence of Y-organ is a prerequisite to ensure the normal progress of vitellogenosis (Meusy et al. 1977), Estrogen was detected in ovaries and freshly spawned eggs of the american lobster Homarus americanus by Donahue, (1940, 1948). Teshima and Kanazawa (1971) reported enzymes that are involved in cleavage of the progesterone side chain to be present in the ovary of Portunus trituberculatus. The crustacean ovary thus seems to have the ability to metabolize steroids. Ovarictomy blocks vitellogenin synthesis in O. gammerella despite the fact that in such females Y-organ, the source of ecdysteroids was intact and active (Junera et al. 1977b). Hence experiment were conducted to know the effect of hormones of reproduction (progesterone and estrodiol) on the enzyme phenol oxidase that hardens the exoskeleton under the influence of ecdysterone. In the present study there was no significant change in the phenol oxidase activity in the haemolymph of the hormones (progestrone and estrodiol) injected animal and the premoult control. However a highly significant decrease in phenoloxidase activity was observed in the exoskeleton (rostrum, carapace, telson and uropod) of progesterone injected animals. In the exoskeleton (carapace, telson, and uropod) of estrodiol injected animals there was a highly significant decrease in enzyme activity, though the enzyme activity per se in the rostrum of estrodiol injected animal was found to be less than that of the control. Due to the effect of progesterone and estradiol whose function is towards reproduction, the enzyme (phenol oxidase) expressed due to the moulting

hormone get retracted from the exoskeleton into the haemolymph to be subsequently carried to the target tissue for getting metabolised into amino - acids.

No significant change in tne enzyme activity was observed in the abdomen of either the control or hormone treated animals. This may be because of the already rising levels of ecdysone which increases synthesis of the exoskeleton hardening enzyme phenol oxidase in premoult animals. Because of the hormones progesterone and estradiol that are injected the further increase in the enzyme level is prevented.

A highly significant increase in phenol oxidase activity was observed in the haemolymph of the soft prawns compared to control. The same trend could be seen in the exoskeleton of the soft prawn. This may be because of the need of the body to produce more phenol oxidase to harden the soft exocuticle of the soft prawns. But in reality inspite of the high enzyme level the exoskeleton remains soft. This may be because of the non-availability of the substrate by certain inhibition of the substrate producing mechanism. Rao et al. (1987) found the rostrum of the soft prawn to be the hardest part of the exoskeleton and a factor for identification of the same. The high enzyme level found in rostrum compared to other region at exoskeleton corroborates his view.

Rostrum being used in the defense mechanism of the prawns, more of the enzyme as well as the substrate is being pumped into that region as a sequence to the body's need to keep the portion hard.

But other parts of exoskeleton are found to be soft. This may be because of the inadequate supply or damage of the substrate production mechanism in those regions. More studies are needed to confirm this view.

5. SUMMARY

5.1 The enzyme phenoloxidase of P. indicus was partially purified from the haemolymph using ammonium sulphate fractionation. A five fold purification with an overall recovery of 52% was achieved. Dialysis was found to result in loss of enzyme activity indicating the presence of dialysible cofactors.

5.2 The rate of the reaction using adrenalin as the substrate was linear upto 3 mts.

5.3 The enzyme was found to have an optimum temperature of 50°C. and optimum pH of 8.5.

5.4 The enzyme had high affinity for the biphenolic substrate adrenalin followed by dopamine, catechol and dopa.

5.5 Monophenolase activity was totally absent.

5.6 The enzyme was found to be stable upto 45°C. but lost 60% of its activity when heated to 70°C for 10 mts.

5.7 The Km and Vmax of the enzyme with adrenalin as the substrate was found to 0.129 and 0.870 /umoles/mg prot /mt. respectively.

5.8 The enzyme was inhibited by copper sulphate and Cupric chloride. Whereas magnesium, manganese and calcium were observed to stimulate the enzyme activity.

5.9 Mercapto ethanol, sodium metabisulphite, ethylene diamine tetra acetic acid and 1,10, phenanthroline severely inhibited the enzyme.

5.10 The hormone betaecdysone has a positive influence on the enzyme level in the haemolymph.

5.11. Betaecdysone increased the enzyme activity in most parts at the exo skeleton.

5.12 No change in enzyme activity was observed in the haemolymph of progesterone and estrodiol injected animals compared to the control group.

5.13 Progesterone and estrodiol significantly decreased the enzyme activity in the exoskeleton.

5.14. In soft prawns the phenol oxidase activity in haemolymph was very high compared to the normal prawns.

5.15 Very high enzyme activity was observed in the exoskeleton of soft prawns.

5.16 The rostrum of the soft prawns showed a high enzyme activity than other parts of the exoskeleton.

REFERENCES

ADELUNG, D. 1967. Zool. Anz. Subbl. 30, 264-272. The Biology of Crustacea Vol. 9., (Bliss, D.E. and Mantel, L.H., eds.). Academic press, Inc. Harcourt Brace Jovanovich, Pub.

ANDERSON, S.O., BARRETT, F.M. 1971. Jr. Insect Physiol. 17, 69-83. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

* ANDERSEN, S.O. 1970. Comparative physiology - Functional aspects of structural materials (ed. Bolis, L. and Schimidt - Nielson K.), pp. 43-48. North Holland, Amsterdam.

* ANTONY P.D., RAJENDRANATHAN NAIR N. 1968. Studies on Phenolase enzymes in Prawns: I - The distribution and physical properties of phenolases in penaeid prawns. Fish. Technol., 5(1) 1968: 31-36.

ANTONY P.D., NAIR M.R. 1975. Studies on phenolase enzyme in prawns: II. The inhibitory action on the enzyme by certain chemical agents. Fish. Technol. 12(2) 1975: 112-115.

* AWAN, F.A. and HOMINICK,W.M. 1982. Observations on tanning of the potato cyst-nematode Globodera rostrochiensis. Parasitology 85, 61-71

BERENDES, H.D. 1967. Gen. Comp. Physiol. 172-176. The Biology of Crustacea Vol. 9., (Bliss, D.E. and Mantel, L.H., eds.). Academi press,Inc., Harcourt Brace Jovanovich, Pub.

BHAGVAT, K. and RICHTER, D. 1938. Biochem. J. 32, 1397.

BHARATHI, N. and RAMALINGAM K. 1983. Electrophoretic study of the enzyme phenol oxidase from the enzyme gland in the foot of Perna virdis. J. exp. Mar. Biol. Ecol. 70, 123-128.

BROWN, C.H. 1952. Quart. J. Micr. Sci. 93, 487-502. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

BRUNET, P.C.J., KENT, P.W. 1965. Proc. R. Soc. (B) 144, 259-274. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

BRUNET, P.C.J. 1967. Selerotins. Endeavour. 26 (98), 68-74. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

BRUNET, P.C.J., COLES, B.C. 1974. Proc. R. Soc. (B) 87, 133-170. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

* BUNKE, D. 1972. Swerotin-Komponenten in den vitellocyten Von- Microdalyellia Fairchildi (Turbellaria). Z. Zellforch. 135, 383-398.

BRUNET, P.C.J. 1980. The metabolism of aromatic aminoacids concerned on the cross linking of inset cuticle. Cited in Manual of research Methods for crustacean Biochemistry and Physiology. CMFRI Special Publication No. 7, 1981 pp. 57. INECT BIOCHEM., 10: 467-500.

BUTENANDT, A., KARLSON,P. 1954. Form. Z. Naturf. (B) 96, 389-391. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

* CAMPBELL W.C. 1960. Presence of Phenolase in the fasciolid metacercarial cyst. J. Parasitol. 46, B48.

CHANG, E.S., SAGE, B.A., and O'CONNOR, J.D. 1976. The qualitative and quantitative determinations of ecdysones in tissues of the crab, Pachygrapsus crassipes, following moult induction. Gen. Comnp. Endocrinol. 30: 21-33.

CHANG, E.S., and O'CONNOR, J.D. 1977. Proc. Natl. Acad. Sci. U.S.A. 74, 615-618. The Biology of Crustacea. Vol. 9., (Bliss, D.E. and Mantel, L.H., eds.). Academic press, In. Harcourt Brace Jovanovich, Pub.

CHANG, E.S., O'CONNOR, J.D., 1978. Gen. Comp. Endocrinol. 36, 151-160. The Biology of Crustacea Vol. 9., (Bliss, D.E. and Mantel, L.H. eds). Academic Press, In., Harcourt Brace Jovanovich, Pub.

DECLEIR, W. and VERCAUTEREN, R. 1965. Cahiers Biol. Marine 8, 101. The Biology of Crustacea Vol. 9., (Bliss, D.E. and Mantel, L.H. eds). Academic Press, In., Harcourt Brace Jovanovich, Pub.

* DECLEIR, W. and VERCAUTEREN, R. 1967. Cahiers Biol. Marine. 8, 101.

DEGENS, E.T., CAREY, F.G., SPENCER D.W. 1967. Nature,Lond.216,601-603 In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

DONAHUE, J.K. 1940. Endocrinology, 27, 149-152. In "Reproductive Biology of Invertebrates". Vol. 1. (K.G.Adiyodi and R.G.Adiyodi eds.) John Wiley and Sons. New York.

DONHHUE, J.K. 1948. Proc. Soc. Exp. Biol. Med. 69, 179-181. In "Reproductive Biology of Invertebrates". Vol. 1. (K.G. Adiyodi and R.G.Adiyodi eds.). John wiley and Sons. New York.

* FAUX, F.R. and MILLS, R.R. 1969. Chem. Commun. 175 - 176. The Biology of Crustacea Vol. 9., (Bliss, D.E. and Mantel, L.H. eds). Academic Press, In., Harcourt Brace Jovanovich, Pub.

FOGAL, W. and FRANEKEL, G. 1969a. J. Insect. Physiol. 15, 1235.

FRAENKEL, G. and HSIAO, C. 1965. J. Insect. Physiol. 11, 513.

GALBRAITH, M.N., HORN, D.H.S., THOMSON, J.A., NEUFELD, G.D. and HACKNEY, R.J. 1969. J. Insect. Physiol. 15, 1225.

GORDON, J. and CARRIKOR, M.R. 1980. Sclerotized protein in the shell matrix of a bivalve mollusc. Mar. Biol. 57, 251-260.

HACKMAN, R.H. and M. GOLDBERG, 1967. The o-diphenol oxidase of fly larvae. J. Insect. Physiol. 18: 581-544.

HAMSHIRE, F., HORN, D.H.S. 1966. Chem. Comm. Z., 37 - 38. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

HOPKINS, P.M., BLISS, D.E., SHEEHAN, S.W., and BOYER, J.R. 1979. Gen. Comp. Endocrinol. 39, 192-207. The Biology of crustacea Vol. 9., (Bliss, D.E. and Mantel, L.H., eds). Academic press, Inc. Harcourt Brace Jovanovich, Pub.

HUBER, R., HOPPE, W. 1965. Chem. Ber. 98, 2403-2424.
In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

*ISHIDA, S. and TESHIROJI, W. 1986. Egg shell formation in polyclads (Turbellaria). Hydrobiologia, 132, 127-135.

JUNERA,H., ZERBIB, C., MARTIN, M. and MEUSY, J.J. 1977b. Gen. and Comp. Endocr., 31, 457-462. The Biology of Crustacea Vol. 9., (Bliss, D.E. and Mantel, L.H., eds.) Academic press, Inc. Harcourt BNrace Jovanovich, Pub.

KALYANI, R., NELLAIAPPAN K. and RAMALINGAM K., 1985. Phenol oxidase activity of reproductive gland and its role in stabilization of egg envelopes of Achatina fulica. Int. J. Invert. Reprod. Devel. 8, 73-81.

KAPLANI , J.N., THOMPSON, M.J., YAMAMOTO, R.T. ROBBINS, W.E., LOULOUDES, S.J. 1966. Steroids 8, 605-623. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A..). Springer-Verlag. Berlin. Heidelberg. New York.

KARLSON, P. and LIEBRAN,H. 1961. Z. Physiol. Chem. 326, 135.

KARLSON, P. and HERRLICH, P. 1965. J. Insect. Physiol. 11, 79-89. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

KARLSON, P. and SEKKRIS, C.E. 1966. Recent Prog. Horm. Res.22, 473-502 In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

KENT, P.W. and BRUNET, P.C.J. 159. Tetrahedron, 7, 252.

* KING, D.S., and SIDDALL, J.B. 1969. Conversion of Alpha ecdysone to Beta-ecdysone by crustaceans and insects. Nature (London) 221, 955-956.

* KNIGHT D.D. 1970. Sclerotization of the perisarc of the calyptoblastic hydroid Laomedea medea. Identification and localization of dopamine in the hydroid. Tiss. Cell. 2, 467-477.

KRISHNAKUMARAN, A. and SCHNEIDERMAN, H.A. 1969. Gen. Comp. Endocrinol. 12, 515-518. The Biology of Crustacea Vol. 9, (Bliss, D.E. and Mantel, L.H., eds.). Academic press, Inc. Harcourt Brace Jovanovich, Pub.

KULKARNI, G.K., NAGABHUSHANAM and JOSHI, P.K. 1979. Effect of progesterone on ovarian maturation in a Marine penaeid prawn P. hardwickii. Indian J. Exp. Biol. 17: 986-987.

LACHAISE,F. and HOFFMANN,J.A. 1977. C.R.Acad.Sci.Paris, 285, 701-704. In "Reproductive Biology of Invertebrates". Vol. 1. (K.G. Adiyodi and R.G.Adiyodi eds.). John wiley and Sons. New York.

LINDA, L. VACCA and MILTON FINGERMAN, 1975. The mechanism of Tanning in the Fiddler Crab, Uca pugillator - 1. Tanning agents and protein carriers in the blood during ecdysis. Comp. Biochem. Physiol., 1975 51B 475-481.

LOCKE.M., KRISHNAN, N. 1971. Tissue and Cell 3, 103-126.
In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

LOWE, M.E. and HORN, D.H.S. 1967. In "Physiological Systems in Semi-arid Environments" (C. Clayton Hoff, Ed.), pp. 155-163.
The Biology of Crustacea Vol. 9., (Bliss, D.E. and Mantel, L.H. eds). Academic Press, In., Harcourt Brace Jovanovich, Pub.

LOWE, M.E., HORN,D.H.S. and GALBRIATH,M.N. 1968.Experientia 24,518-519
The Biology of Crustacea Vol. 9., (Bliss, D.E. and Mantel, L.H. eds). Academic Press, In., Harcourt Brace Jovanovich, Pub.

* LOWRY, C.H., ROSE BROUGH, N.J., FARR, A.I. and RANDAL, R.J. 1951. Protein measurement with Folinphenol reagent. J. Biol. Chem., 193-265.

MALEK, S.R.A. 1961. Nature, 198, 301. The Biology of Crustacea Vol. 9, (Bliss, D.E. and Mantel, L.H. eds). Academic press, Inc. Harcourt Brace Jovanovich, Pub.

MANSOUR T.E. 1958. Effect of serotonin on Phenol oxidase from the liver Fluke Fasciola hepatica and from other sources. Biochim. Biophys. Acta 30, 442-500.

MASAAKI ASHIDA and KENNETH SODERHALL, 1984. The prophenol oxidase activating system in cray fish. Comp. Biochem. Physiol. 77B (1) 21-26.

MASON H.S. 1955. Comparative biochemistry of the phenolase complex. Adv. Enzym. Relat. Subj. Biochem. 16. 105-185.

* MEENAKSHI,V.R. and SCHEER, B.T. 1961. Comp. Biochem. Physiol. 3, 30.

MEUSY, J.J., BLANCHET, M.F., JUNERA, H. 1977. Gen. Comp. Endocr. 33, 35-40. In "Reproductive Biology of Invertebrates". Vol. 1. (K.G. Adiyodi and R.G.Adiyodi eds) John Wiley and Sons, New York.

MILLS, R.R. 1967a. J. Insect. Physiol. 13, 815.

MILLS, R.R., LAKE, C.R. and ALWORTH, W.L. 1967b. J. Insect Physiol. 13, 1539.

MILLS,R.R. ANDROVNY, S. and FOX F.R. 1968. J.Insect Physiol.14,603-611. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

* MONNE L. and HOING, G. 1954. On the properties of coccidian oocysts. Ark. Zool. 7, 251-255.

NAGABHUSHANAM, R., JOSHI, P.K. and KULKARNI, G.K. 1982a. Induced spawning in Parapanaeopsis stylifera (H. Milne Edwards) using a steroid hormone, 17-Hydroxy-progesterone. Proc. Symp. Coastal Aquaculture, 1982, 1: 37-39.

NAGABHUSHANAN, R. and KULKARNI, G.K. 1982. Endocrine regulation of reproduction in the marine female prawn Parapenaeopsis hardwicki (Miers). Proc. Symp. Coastal Aquaculture, 1982, 1: 40-47.

NAKANESHI, K., KOREEDA, M., CHANG, M.L. and HSU, H.Y. 1968. Tetrahedron letters N. 9, 1105. In 'Chemical zoology' Vol. 6. Arthropoda, part B (Florkin, M. and Scheer, T.B. eds). Academic Press, New York and London.

NELLAIAPPAN K., THANGARAJ T., RAMALINGAM K., 1982. Phenol oxidase activity and its role in cuticular sclerotization in a mole crab Emirita asiatica Milne Edwards. J. Exp. Mari. Biol. Ecol. 61: 75-83.

NELLAIPPAN K., VINAYAKAM A., KALYANI, R. 1989. Electrophoretic Paltrin of Blood and Cuticular Phenol oxidase of different crustacean Experimental Biol. Vol. 48 No.(3): p. 177.

NELLAIPPAN K. and KALYANI R. 1989. Mantle phenoloxidase and its role in sclerotization in a Snail Achatina fulica cited in Arch. Intern. Physiol. Biochem 97, 45-51.

NELLAIAPPAN K., FATIMA A. and DHANDAYUTHAPANI I.S. 1989. The properties of Phenol oxidase in Fasciola gigantica. Parasitology 99, 403-407.

PAU R.N., and ACHESON, R.M. 1968. Biochem. Biophys.Acta 158, 206-211 In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

PAU R.N, and EAGLES PAM 1975. The isolation d an o-diphenol oxidase from third-instar larvae of the blowfly Calliphora erythrocephala. Biochem J. 199: 707-712.

PINHEY K.G. 1930. J. exptl. Biol 7, 19. The Biology of Crustacea Vol. 9., (Bliss, D.E. and Mantel, L.H., eds.). Academic press, inc. Harcourt Brace Jovanovich, Pub.

* PRESTON, J.W. & R.L.THYLOR, 1970. Observations on the phenol oxidase system in the haemolymph of the cockroach leucophea maderae. J. Exp. Zool., 71, 489.

PRAYOR, M.G.M. 1940. Proc. R. Soc. (B) 128, 378 - 393.
In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

PRYOR, M.G.M. 1940b. Proc. Roy. Soc. B 128, 393 - 407.
In "Reproductive Biology of Invertebrates". Vol. 1. (K.G. Adiyodi and R.G.Adiyodi eds.). John wiley and Sons. New York.

RZEPECKI, L.M., CHIN. S.-S., WAITE J.H., LAWIN, M.F., 1991. Molecular diversity of marine glues: poly phenolic proteins from Five mussel species. Mol. Mar. Biol. Biotechnol 1(1) Sep. 1991 pp. 7-88

*SAMATA, T., T. SANGUANSRI, D. CAZAUX, C., HAMM, M., ENGELS, J. and KRAMPITZ, G. 1980. Biochemical studies on the components of mollusc shells. In Mechanisms of Biomineralization in animals and plants (ed. by Omori M. & Watabe H.) pp. 37-47. Tokai University press Tokyo.

SCHLOSSBERBER-RAECKE, I., KARLSON, P. 1964. J. Insect. Physiol. 10, 261-266. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. New York.

*SEED J.L., BOFF M. and BENNET J.L. 1978. Phenol oxidase activity induction in Female Schistosomes by an in vitro incubation. 97B(1) 1990. J. Parasit. 64, 283-289.

SEED. J.L. and BENNETT J.J. 1980. Schistosoma manosoni : Phenol oxidases role in egg shell formation. Expl. Parasit. 49, 430-441.

SELIGMAN, M., FRIEDMAN, S., and FRAENKEL, G. 1969. J. Insect Physiol. 15, 553 and 1085.

*SIMPSON, B.K., MARSHALL, M.R., OTWELL, W.S. 1988. Phenol oxidases from Pink and White shrimp: Kinetic and other properties. Cited in: 2802-3Q6. ASFA Aquaculture abstracts 6(3): 1989. J. Food. Biochem. 12(3) 1988. pp. 205-217 En. Cn.

SIU-MING CHAN, SUSAN, M. RANKIN and LARRY L. KEELEY, 1990. Effect of 20-hydroxy ecdysone injection and eyestalk ablation on the moulting cycle of the shrimp Penaeus vannamei. Comp. Biochem. Physiol. 96A(1): 205-209.

SKINNER, D.M. and GRAHAM, D.E. 1970. Science 169, 383-385.
The Biology of Crustacea Vol. 9., (Bliss, D.E. and Mantel, L.H. eds). Academic Press, In., Harcourt Brace Jovanovich, Pub.

* SMITH, V.J., SOEDDERHAELL, K. A comparison of phenol oxidose activity in the blood of marine invertebrates. Cited in 2620-1Q22. ASFA. Part 1 22 (2) 1992. DEV. Comp. Immunol. 15(4) 1991. pp. 251-261 En; en.

SMYTH J.D. 1954. A technique for the histochemical demonstration of polyphenol oxidase and its application to egg shell formation in helmintnus and byssus formation in mygilius. Cited in Comp. Biachem. & Physiol. 97B (1) 1990. J. Micros. Sci. 95. 139-152.

* SMYTH, J.D. and CLEGG, J.A. 1959. Eggshell formation in trematodes and cestodes. Expl. Parasit. 8, 286-323.

STAY, B., ROTH, L.M. 1962. Ann. ent. Soc. Am. 55, 124 - 130. In "Zoophysiology and Ecology". Vol. 4/5. Biology of the Arthropod Cuticle (Neville, A.C.). Springer-Verlag. Berlin. Heidelberg. Newyork.

SUMNERS, N.M. 1967. Cuticle sclerotization and blood phenol oxidose in the fiddler crab, Uca pagnax. Comp. Biochem, Physiol., 23: 129-138.

TESHIMA, S.I. and KANAZAWA, A. 1971. Bioconversion of progesterone by the ovaries of the crab, portunus, tribuberculatus. Gen. Comp. Endor., 17, 152-157.

THANGARAJ, T., NELLAIAPPAN, K. and RAMALINGAM K. 1907. Comparative studies on cutialar and blood phenol oxidases of a shorecrab Ocypod platytarsis. Arch. Inst. Physiol. Bioch. 90: 63-68.

* TIDBALL, J.G. 1982. Ultrastructural and cytochemical analysis of the cellular basis or tyrosine derived collagen cross links in eptogorgia virgulata (Cuiclaria). Cell. Tiss. Res. 222, 635-645.

VACCA, L.L. and M. FINGERMAN, 1975. The mechanism of tanning in the fiddler crab Uca pugilator II. Thje cyclic appearance of tanning agents and attached carrier proteins in the blood during moulting cycle. comp. Biochem. Physiol. 51B: 483-487.

VEDAAVYARA RAO, P. 1987. Soft prawn in Syndrome in prawns. R and D series for Marine Fishery resources Management.

VINCENT J.F.V. and HILLERTON J.E. 1979. The tanning of insect cuticle - a critical review and revised mechanism. J. Insect. Physiol. 25, 653-658.

WAITE, J.H. and WILBUR K.M. 1976. Phenol oxidase in the periostracum of the marine bivalve geukensia (Modiolus) demiss (Dillwyn). J. rab. Zool. 195 358-368.

* WAITE, J.H. 1983a. Adhesion in byssally attached bivalues. Biol. Rev. 58, 209-231.

WAITE, J.H., 1983b. Quinnone-tanned sclero proteins. In the mollusca (ed. by Wilbur K.M.), Vol. 1 pp. 467-504. Academic Press, N.Y.

WAITE, J.H. 1985. Catechol oxidase in the byssus of the common mussel Mytillus edulis J. mar. Biol. Ass. U.K. 65, 359-371.

* WAITE, H.J. 1990. the phylogency and chemical diversity of quinone-tanned glues and varnishes. Comp. Biochem. Physiol. 97B 19-29.

* WHEELER, A.P., RUSENKO, K.W., SWIFT, D.M. and SIKES, C.S. 1988. Regulation of in vitro and in vivo at $CaCo_3$ crystallization by fractions of oyster shell matrix. Mar. Biol. 98, 71-80.

WHITEHEAD, D.L. 1969. Nature 224 721.

YAMAZAKI, H.L. 1969. The cuticular phenoloxidases in Drosophila virilis. J. Insect. Physiol. 15, 2203-2211.

* Not referred in original.