Activation Mechanism of Pre-Phenoloxidase in Lobster and Shrimp

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Phenoloxidase in shrimp and lobster is shown to exist in latent form which could be activated by trypsin and by an endogenous enzyme with tryptic activity. On Sephadex G-100 gel, three isoenzymes, differing in molecular weights, were isolated from naturally activated lobster shell extracts. Mechanism of activation of pre-phenoloxidase involving limited proteolysis by the activating enzyme to form isoenzymes has been proposed.

Phenoloxidase (EC 1.10.3.1-*o*-diphenol: oxidoreductase), a copper protein catalysing *o*-hydroxylation of monophenols and dehydrogenation of *o*-diphenols (Mason, 1955), is responsible for browning in certain fruits and vegetables and blackening or melanosis in crustaceans. While browning has been extensively studied in fruits (Joslyn & Pointing, 1951) only limited studies have been carried out on melanosis with respect to its localization (Kakimoto & Kanazawa, 1956; Bailey *et al.*, 1960; Antony & Nair, 1968) and prevention (Faulkner *et al.*, 1954; Bailey & Fieger, 1954; Ito, 1967). The enzyme responsible for melanosis itself has received much less attention except for a few studies on its properties in partially purified systems (Bailey *et al. loc cit.*, Ito *loc cit.)*.

Phenoloxidase activity in shrimp *(Penaeus setiferus, Penaeus aztecus)* increases on heating at 40 to 60°C (Bailey *et al., loc cit.)* and on short exposure to air temperatures prior to icing (Fieger *et al.*, 1958). Though this has been attributed to pro-enzymic nature of phenoloxidase with its active centres getting exposed at higher temperatures (Bailey *et al., loc cit.*), existence of zymogen has not been established. Absence of such zymogen is evident in *Pandulus jordani,* another Pacific shrimp (Flores & Crawford, 1973).

It is not known whether multiple forms of phenoloxidase exist in shell-fish system as in mushroom, potato, hamster, apple and peach (Constantinides & Bedford, 1967; Pomerantz, 1963; Horowitz *et al.,* 1960).

This paper presents a number of experiments providing evidence for existence of phenoloxidase in penaeid shrimp and spiny lobster (*Panulirus homarus* Linn) in zymogen form and discusses possible mechanism of activation involving an activating proteolytic enzyme.

Materials and Methods

Fresh shrimp and live spiny lobster used in the experiments were caught off Bombay coast.

Extraction of enzyme

Lobster and shrimp shells and cephalothorax were homogenized separately with 0.02 M potassium phosphate buffer of pH 7.4 in a Waring Blender for 2 min. Homogenates (25%) were strained through muslin cloth and centrifuged at 10,000 *g* for 10 min and the supernatants were used for enzyme assay. All these operations were completed at 0–4°C. Such preparation contained 20–25 mg protein per ml extract.

Assay of enzyme activity

Enzyme was assayed by the method of Horowitz *et al. (loc cit.)* with some

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modifications. The assay system consisted of 0.2 ml of enzyme preparation and 2.8 ml of 0.05 M potassium phosphate buffer of pH 6.5 containing 2 mg DL-dopa (Fluka AG, Buchs SG, Switzerland) giving a final concentration of 0.33×10^{-2} M in reaction mixture. The formation of dopachrome at 25°C was measured at 470mμ using B & L Spectronic-20 colorimeter. Using a zero blank, change in optical density (OD) in 5 min was expressed as relative activity and specific activity was taken as change in OD per mg protein in five minutes at 25°C.

Protein determination

Protein concentration in the extracts was measured by the Biuret method at 540 mμ using B & L Spectronic-20 colorimeter.

Effect of ice storage on phenoloxidase activity in shrimp and lobster

Enzyme extracts from cephalothorax and shells of shrimp and lobster were held in ice, with or without the addition of toluene, and the activity measured at intervals. Enzyme activity was also determined in shells from shrimp and lobster held intact in ice for different periods prior to extraction.

Effect of pH and storage temperature on phenoloxidase activity in lobster

1 ml aliquots of aqueous extract of fresh lobster shell, mixed with 1 ml portion of 0.1 M citrate (pH 4.6 to 5.45), potassium phosphate (pH 5.7 to 7.9) and tris-HCl (pH 8.05 to 9.0) buffers, were stored in ice for 24 h before the enzyme activity was measured. Lobster shell extracts held at 0, 10, 20 and 30°C were assayed for phenoloxidase activity at intervals.

Change in phenoloxidase activity in subcellular fractions of lobster shell extract during storage

Differential centrifugation of 25% shell homogenate from fresh lobster in 0.025M sucrose solution was carried at 2°C using 3 speeds (750*g*-10 min, 10,000 *g*-10 min and 100,000*g*-60 min). Respective pellets (P) and supernatants (S) were held at 0°C and phenoloxidase activity in different fractions was determined at 0 and 48 h. Similarly, enzyme activity was determined in various fractions obtained by differential centrifugation of shell extract prepared from lobster stored intact for 48 h in ice.

Activation effect of various treatments on lobster pre-phenoloxidase

Boiled extract–fully activated prior to boiling (0.2 ml), Triton x 100 (0.04 ml of 10%), Tween–80 (0.01 ml), cupric ions (10 to 100μg of $CuSO_4$ $5H_20$), trypsin (Nutritional Biochemicals Corporation, USA) (100μg), chymotrypsin (Worthington Biochemicals Corporation, USA) (100μg) and pepsin (E. Merck, Germany) (100μg) were separately added to enzyme extract (0.2 ml) and the relative activity determined. Effect of repeated (6 times) freeze-thawing on the activity was also determined.

Trypsin induced activation of pre-phenoloxidase

Effect of incubation period on the trypsin induced enzyme activation was determined by holding enzyme preparation (0.2 ml, pH 7.4) with trypsin (100μg) at 25°C for upto 20 min. Concentration effect was measured by adding upto 200μg trypsin to the assay system. Soyabean trypsin inhibitor (SBTI) (Worthington Biochemicals Corporation, USA) (100μg) was added to the assay system before and 2 min after the addition of trypsin (100μg) and the activity determined.

Effect of SBTI on pre-phenoloxidase activation during storage

0 to 200μg SBTI was added to 0.2 ml enzyme preparation which was then stored at 3°C for 4 h and assayed.

Ammonium sulfate fractionation

Lobster shell extract, centrifuged in cold at 10,000 *g* for 10 min, was brought

to 40% saturation using solid ammonium sulfate. The precipitated proteins were centrifuged out at 10,000*g* for 10 min and extracted with 0.02M phosphate buffer of pH 7.4 using a hand homogenizer. Insoluble proteins were removed by centrifugation at 10,000*g* for 10 min. By continued precipitation 40–50, 50–60, 60–70% saturation ammonium sulfate fractions were prepared.

Sephadex gel filtration

Ammonium sulfate fractions (10 ml each of 75% saturation with 10 mg protein/ml) prepared from lobster shell extracts, one stored for 72 h at 0°C without trypsin and the other stored for 24 h at 15°C with trypsin (0.5mg/ml), were applied on Sephadex G-100 (Pharmacia, Uppsala) column (1.7 x 55 cm) equilibrated with 0.02M phosphate buffer of pH 7.4 and eluted with the same buffer at a flow rate of 22 ml per h. Enzyme activity in the fractions (70 x 3 ml) and their optical density at 280mμ were measured. Lobster shell extract, held at 0°C for 3 h and frozen for 3 days, was subjected to ammonium sulfate fractionation and Sephadex gel filtration. Phenoloxidase activity was determined in the fractions with and without trypsin activation (100μg/0.2 ml fraction) at 25°C for 3 min.

Measurement of proteolytic activity

Ammonium sulfate fraction, activating pre-phenoloxidase, was tested for proteolytic activity by the method of Kunitz as described by Laskowski (1955), with slight modifications. Hemoglobin (Sigma Chemical Co., USA), bovine albumin (Sigma Chemical Co., USA) and casein (Calbiochem, USA) 0.05% in 0.1M sodium phosphate buffer (pH 7.6) were used as substrates. Proteolysis was followed by measuring the released trypsin by Miller's (1955) method.

Purification of pre-phenoloxidase activating enzyme

Lobster shell extract (25%) was subjected to ammonium sulfate fractionation (0–30% saturation, twice), Sephadex G-200 column chromatography and DEAE-Sephadex A–50 column chromatography. Proteolytic activity and prephenoloxidase activating activity were determined after each purification step.

Results and Discussion

Phenoloxidase activity in extracts from lobster cephalothorax and shell (Fig. 1) increased by 9 and 40 fold respectively during icestorage for 4 days, indicating

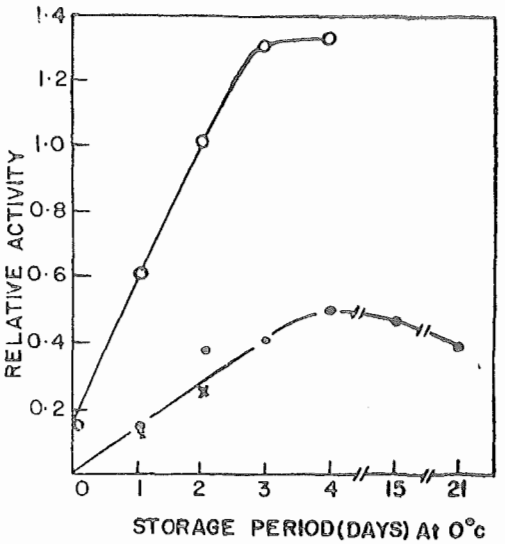

Fig. 1. *Effect of ice storage on phenoloxidase activity in fresh lobster*

X—X Cephalothorax extract

●—● Shell extract

○—○ Shell extract from stored lobster

activation of the enzyme. The activity increased even in the presence of toluene showing the activation to be non-bacterial. The activity increasing to maximum in 2 to 4 days declined slowly thereafter, still remaining quite high for upto 21 days. Change in the activity in extract was similar to that in stored intact lobster. Similar results were obtained with shrimp, cephalothorax and shell extracts showing respectively 26.5 and 500% increase in activity in 2 days at 0°C. Cephalothorax in both shrimp and lobster contained good amount of free activity as compared to shell. Hence lobster shell with low free activity and high

specific activity appeared better suited for studies on mechanism of activation.

Phenoloxidase activity increased with storage temperature and also rose to higher levels with it (Fig. 2). Activity at 20 and 30°C reached its maximum in 4 to 5 h while at 10 and 0°C it took 22 and 48 h respectively. The level of maximum activity reached, correlated fairly well

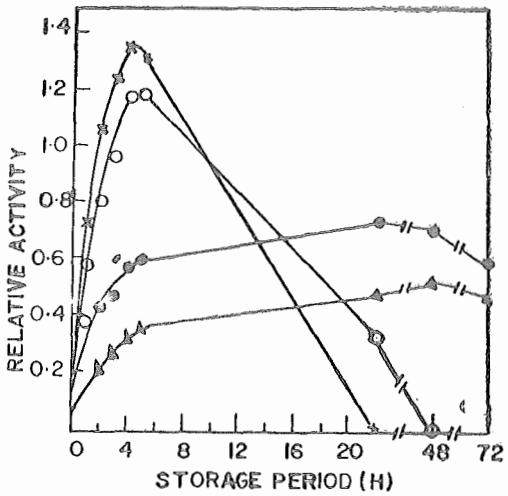

Fig. 2. *Effect of storage temperature on phenoloxidase activity in shell extract of fresh lobster*

◀—◀ 0°C; ●—● 10°C;

o—o 20°C; ×—× 30°C

with temperature of storage. At 20 and 30°C the activity started decreasing after about 4–5 h and was completely lost in 48 and 22 h respectively, showing temperature dependence of the process. This loss of activity at high temperature could be due to proteolytic and bacterial degradation. It was observed that intensity of blackening in the extract was the most at 10°C followed in order by 0,20 and 30°C. While less blackening at 0°C is due to slow melanosis, that at higher temperature could be due to destruction of phenoloxidase itself.

Optimum pH, for activation of the pre-enzyme was found to be 8.4 in tris buffer at 0°C.

Phenoloxidase activity in subcellular fractions during storage (Table 1) increased only in soluble fraction which accounted for 87.9% of the total activity at 48 h indicating the presence of pre-enzyme in soluble form. This also ruled out the possibility of solubilization effect for the increased activity.

Repeated freeze-thawing, Triton x 100 and Tween-80 were not effective in activating the enzyme. Cupric ions increased the activity (25%) but not to the extent to account for observed activation during storage. This, together with the absence of activation effect by boiled extract ruled out the possibility for metal ion induced activation.

Addition of trypsin instantaneously increased the activity by 8.5 fold, suggesting proteolytic removal of an inhibitor or activation by the direct action of trypsin on pre-phenoloxidase molecule. SBTI added prior to the addition of trypsin, retarded the trypsin induced activation, but when added 2 min after the addition of trypsin, had no effect implying the proteolytic nature of the reaction taking place at a fast rate and involving active site of trypsin. SBTI added to the shell extract, however, did not prevent the activation of the enzyme during storage. Chymotrypsin and pepsin, had no activating effect on the enzyme denoting the specificity of the process of activation. Silkworm pre-phenoloxidase is reported to behave in similar way with respect to chymotrypsin (Ohnishi *et al.*, 1970).

Phenoloxidase activity increased with trypsin concentration and incubation period (Figs. 3a and 3b). Slow rate of increase in the activity at lower concentrations of trypsin may be due to the use of crude extracts for these studies and may also be due to the lag shown by phenoloxidase at low concentrations, as observed during the present investigation. Apart from this slow period, the activation increased progressively with trypsin concentration. Absence of a lag period prior to the activation (Fig. 3b) and proportional activation with concentration of trypsin (Fig. 3a), suggested that the activation is the result of direct action of trypsin on the pre-enzyme molecule and hence could be of the endogenous activator too.

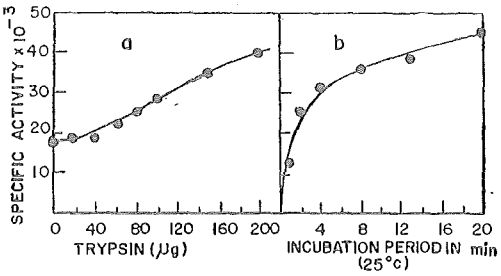

Fig. 3a & b *Effect of trypsin concentration and incubation period on pre-phenoloxidase activation*

Tests on different ammonium sulfate fractions for the presence of pre-phenoloxidase and its activator revealed the presence of pre-phenoloxidase in 40-50% saturation fraction and of an activating factor in 0–40% saturation fraction. Discovery of the activating factor in ammonium sulfate fraction, which lost activating function on heating, strongly suggested the enzymic nature of the endogenous activator.

The activator fraction (0-40 ammonium sulfate fraction) hydrolysed casein efficiently (Table 2) showing its proteolytic nature. However, it hydrolysed hemoglobin, bovine albumin and cytochrome C only at random.

On Sephadex G-100 gel chromatograms (Fig. 4) three fractions possessing

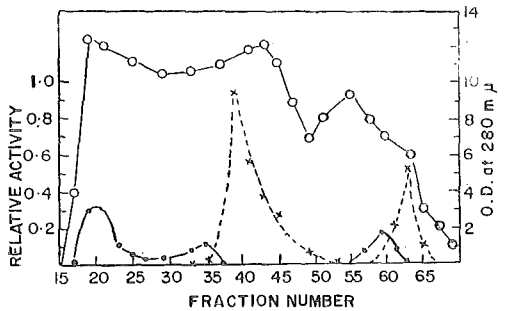

Fig. 4. *Sephadex G-100 chromatography of phenoloxidase activated with and without trypsin*

O—O O.D at 280 m µ;

X—X Trypsin activated;

●—● Activated without trypsin

phenoloxidase activity emerged from ammonium sulfate fraction of the extract activated by endogenous activator and two fractions emerged from that activated by trypsin. All the five fractions differed in molecular weight. These results established the presence of multiple forms of phenoloxidase in lobster, indicated proteolytic nature of the activation process and pointed out the possible difference in the sites hydrolysed by endogenous enzyme and trypsin.

From Fig. 5 showing free and latent phenoloxidase activity in partially activated extract it is clear that the molecular weights of the pre-and active-phenoloxidase are almost identical and the two low

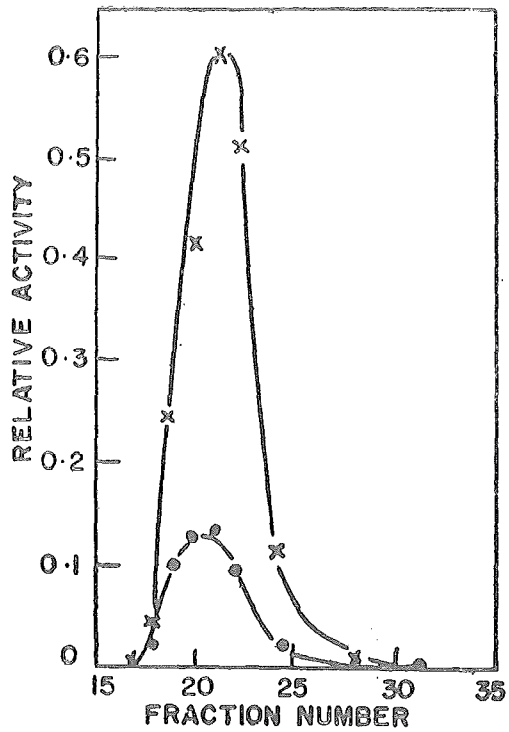

Fig. 5. *Sephadex G-100 chromatography of partially activated phenoloxidase*

●—● Free activity

X—X Total activity

molecular weight enzyme fractions are absent at this stage of activation, suggesting sequential breakdown of pre-enzyme to form isoenzymes.

Table 1. *Change in phenoloxidase activity in subcellular fractions during storage at 0°C*

Fraction	Specific activity (change in OD/ mg protein/5 min at 25°C)	Percentage of the total free activity*	Specific activity (change in OD/mg protein/ 5 min at 25°C)	Percentage of the total free activity*
P-750 x *g*	0	0	4.1	3.3
P–10,000 x *g*	0.5	0.4	0.4	0.3
P–100,000 x *g*	10.8	8.7	10.5	8.5
Soluble fraction–100,000 x *g*	32.0	25.8	109.0	87.9

*Total free activity is total specific activity in all the fractions after 48 h (=124)

Table 2. *Proteolytic activity of activator fraction*

Protein	mμM tyrosine released 0 min	30 min	60 min	120 min
Hemoglobin	0.0	0.0	0.58	2.9
Bovine albumin	0.0	0.0	1.74	1.16
Cytochrome C	0.0	0.0	1.74	0.58
Casein	0.0	10.05	18.84	39.50

Table 3. *Proteolytic and pre-phenoloxidase activating activities at different stages of purification of activating enzyme*

Stage of purification	Proteolytic activity* mμM tyrosine released/ mg protein A	Pre-phenoloxidase activating activity units/mg protein B	A/B
Crude extract	5.3	2.16	2.46
1st Amm. sulfate fractionation (30% sat.)	16.8	7.65	2.20
2nd Amm. sulfate fractionation (30% sat.)	18.6	11.0	1.69
Sephadex G–200 column chromatography	30.5	14.7	2.07
DEAE–Sephadex A–50 column chromatography	39.0	16.8	2.32

* Casein is used as the substrate

The ratio of proteolytic activity to pre-phenoloxidase activating activity at different stages of purification remained fairly constant (Table 3) indicating single protein to be responsible for both the activities.

The studies thus establish the presence of phenoloxidase in shrimp and lobster in latent form getting activated by an endogenous proteolytic enzyme giving rise to iso-enzymes. The plausible mechanism of activation of pre-phenoloxidase in lobster would be that the pre-enzyme in presence of activating enzyme loses by limited proteolysis a small portion of the molecule, without significantly affecting the molecular weight. This leads to conformational change in the molecule, exposing the active centres with the appearance of phenoloxidase activity. Thus the high molecular weight iso-enzyme is formed. Further on storage, selective proteolytic removal of peptides, not involved in the catalytic activity, continues giving rise to iso-enzymes with lower molecular weights. It is also possible for the pre-enzyme to lose a part of the molecule without getting activated. Similar mechanism of pre-phenoloxidase activation may be operative in shrimp.

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