

Properties of phenoloxidase from haemocytes of *Scylla tranquebarica*

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ABSTRACT

The presence of phenoloxidase (PO) activity in the haemocytes of *Scylla tranquebarica* was electrophoretically and spectrophotometrically studied. Majority of the enzyme was located as proenzyme, prophenoloxidase (proPO) in the haemocytes. The enzyme prefers L-dihydroxyphenylalanine (L-dopa) as its substrate than phenol and is optimally active at pH 8.0. Besides trypsin, the proPO was also activated by both Gram positive and Gram negative microbes *in vitro* while, chemicals such as sodium azide, thiourea and EDTA significantly inhibited the enzyme expression. The protein needs considerable levels of divalent cations like calcium (20 mM as CaCl₂) or magnesium (20–50 mM as MgCl₂) for its activity. The gel filtration chromatography of haemocyte lysate supernatant showed a single major peak of protein having PO activity. Electrophoresis of purified PO by native PAGE revealed a single prominent band of approximately 167.2 kDa which was further resolved to three bands having molecular mass of approximately 77.1, 56.9 and 30.2 kDa respectively, on SDS-PAGE.

1. INTRODUCTION

Decapods utilise two broad categories of defence responses against invading pathogens namely, cellular and humoral. Cellular immune response functions for encapsulation, phagocytosis and nodule formation [1] while the antibacterial proteins, clotting system and prophenoloxidase (proPO) system belongs to humoral immune responses [2, 3]. The prophenoloxidase system is considered as an important mechanism of innate defence in arthropods. Its active form phenoloxidase (PO), a copper containing enzyme, catalyzes two successive reactions; hydroxylation of a monophenol to o-diphenol and oxidation of o-diphenol to o-quinone and initiate the synthesis of melanin that will act as a toxin against microorganisms [4]. The location of proPO is highly varied among arthropods. However, in decapod crustaceans, proPO is located mainly in granular haemocytes and haemocyte lysate supernatant was used as the enzyme source [5-8]. Although, the presence of a proPO system involved in non-self recognition is well documented in decapods, no such reports are available for *Scylla tranquebarica*. Therefore the objective of this paper was

to study the main biochemical properties of proPO from haemocytes of *S. tranquebarica*.

2. MATERIALS AND METHODS

2.1. Animals

Crabs, *Scylla tranquebarica* (7.5 ± 0.5 cm; 60 ± 1.9 g) were collected from Muttukkadu creek, Chennai, India. Twenty crabs were maintained in two-ton fibreglass tanks containing seawater (pH: 8.3 ± 0.2; salinity: 26 ± 1.0 ppt; temperature: 25 ± 1.0 °C) for an acclimatization period of 20 days. The water was aerated continuously and changed once a day. The animals were fed with boiled clam meat during the period of study. Only healthy crabs, with no missing limbs and carapace lesions were used for the experiment.

2.2. Haemocyte lysate preparation

The animals were bled, after surface sterilization with 70 % ethanol, directly from the sinuses of the appendages using 26 gauge needle fitted to sterile 2 ml plastic syringe containing anticoagulant solution (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 10 mM EDTA, pH 7.2) at 1:1 ratio.

The hemolymph was transferred to 1.5 ml eppendorff tubes and held on ice cubes, then centrifuged at 700 g for 10 min at 4 °C and pellet was separated. The supernatant, plasma collected separately also used for detecting the PO activity.

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The cell pellets were resuspended in sodium cacodylate buffer, CAC1 (10 mM sodium cacodylate, 0.45 M NaCl, 20 mM CaCl₂, 30 mM MgCl₂, pH 8.0) and homogenised. The resultant solution was centrifuged at 16,000 g for 45 min at 4 °C. The supernatant designated as haemocyte lysate supernatant (HLS) was stored in aliquots at 4 °C, -4 °C and -30 °C separately and used as enzyme source for further study.

2.3. Determination of phenoloxidase activity

PO activity was measured by recording the formation of dopachrome from L-dopa from HLS and plasma [9]. Briefly, 50 µl of HLS was incubated with same quantity of trypsin (2 mg ml⁻¹ of CAC1 buffer) in 96 well flat bottom microtitre plate (Tarsons) for 20 min at 25 °C. Then, 100 µl of L-dopa (3 mg ml⁻¹ in CAC1 buffer) was added. Absorbance was measured after 5 min at 490 nm using a MULTISCAN ELISA Reader (Labsystems, Finland) for 20 min at every 2 min intervals. PO activity is expressed as units; one unit of enzyme was defined as the increase in absorbance of 0.001 min⁻¹ mg⁻¹ of protein. The concentration and quantity of trypsin and L-dopa used for the assay of PO throughout the present study was same unless specified.

2.4. Substrate specificity and K_m determination for PO activity

Substrate specificity and K_m determination was employed for L-dopa and phenol. For this, 50 µl of HLS was first incubated with trypsin for 20 min at 25 °C and then PO activity was measured by adding 100 µl of substrates at various concentrations (0.5, 1, 2, 5, 10, 15, 20, 25 and 30 mM) into it. The reaction velocity was calculated from Lineweaver-Burk plots. The kinetic method at maximum absorbance (V_{max}) was used to determine the velocity of the reaction. The K_m for L-dopa and phenol was calculated from the result of the experiment.

2.5. Effect of pH on PO activity

To determine the optimum pH required for PO activity, 50 µl of HLS was incubated with trypsin and 100 µl of L-dopa (3 mg ml⁻¹), dissolved in phosphate buffer having different pH (3.0 to 8.5 with 0.5 increment) was added and absorbance was measured at 490 nm. The pH having the highest activity was considered as optimum pH for PO from haemocytes of *S. tranquebarica*.

2.6. Effect of exogenous elicitors on PO activity

The elicitors used in the present study were: (a) buffer control (CAC1) (b) trypsin (c) different Gram-negative bacteria of *Vibrio* spp viz., *Vibrio cholerae*, *V. parahaemolyticus*, *V. mimicus*, *V. fluvialis*, *V. harveyi*, *V. mediterrani*, *V. logei*, *V. campbellii*, *V. alginolyticus*, *V. fisherii*, *V. vulnificus* and (d) a Gram-positive bacteria, *Streptococcus fecalis*. All microbes were isolated and identified [10] from shrimp, *Penaeus monodon*, collected from culture ponds in Mahabalipuram, Tamil Nadu (India). All bacteria were grown in Brain Heart Infusion broth with 1.5 % NaCl at 37 °C and cells were harvested by centrifugation at 5000 g for 20 min at 4 °C. The bacterial pellet was washed and centrifuged twice with sterile PBS. The final pellet was resuspended in sterile PBS and

made the final concentration of 10⁷ cells ml⁻¹. For detecting the enzyme activity, 50 µl of HLS was incubated with 50 µl of different elicitors for 20 min at 25 °C. The PO was then detected using L-dopa as above.

2.7. Effect of inhibitors on PO activity

The compounds tested for inhibition assay were: thiourea, sodium azide and EDTA. To 50 µl of HLS, an equal quantity of trypsin was added and incubated for 20 min. Subsequently, 50 µl of 10 mM inhibitor solutions were added and incubated for 30 min at 25 °C. Finally, 100 µl of L-dopa was added and PO activity was measured. A control set with 50 µl of CAC1 buffer was also used instead of the inhibitor solution for phenoloxidase activity. Percentage reduction of PO activity was calculated between the trypsin treated and samples having inhibitors.

2.8. Calcium and magnesium dependence on PO activity

In order to study the calcium and magnesium dependence on PO activity, HLS was prepared with CAC2 buffer (10mM sodium cacodylate). Fifty µl of the prepared HLS were incubated with trypsin for 20 min and divided into two groups. To the first group, 50 µl of 5, 10, 20, 50 and 100 mM CaCl₂ was added while in the other group, same quantity of MgCl₂ was added and incubated for 20 min. PO activity was measured after adding L-dopa. The concentration of divalent cation that shows the highest PO activity was selected as optimum level required for the assay.

2.9. Storage stability

To study the storage stability at different temperatures, the prepared HLS was kept at 4, -4 and -30 °C. PO was determined after 7, 14, 21, 28 and 35 days. The percentage reduction of PO was calculated by comparing its activity with that of initial day (73.14 ± 2.25 units).

2.10. Enzyme purification by gel filtration chromatography

Three ml of HLS containing 140 mg ml⁻¹ of protein was applied to a Sephadex G 200 (Pharmacia, Sweden) column (30 X 2 cm) equilibrated with CAC1 buffer. The column was eluted with the same buffer at a flow rate of 1 ml min⁻¹, the first 30 ml was discarded and then 3 ml fractions were collected. Protein concentrations and PO was determined for each fraction. The fractions showing high proPO activity were pooled and concentrated by dialysis bag (Hi-Media) with 10 kDa molecular cut off against sucrose followed by equilibration with distilled water. The samples were then stored at -30 °C until further study.

2.11. Polyacrylamide gel electrophoresis (PAGE) of partially purified proPO

Polyacrylamide gel electrophoresis of the partially purified proPO was performed using 10 % gels and Tris-glycine buffer (pH 8.3), run under native and denaturing conditions [11]. For native PAGE, the gel was run at 130 V for 4 h and molecular weight markers from 44 to 205 kDa were included. SDS-PAGE

was run at 80 V for 3 h, using protein from 14 to 97.4 kDa as molecular weight markers. To observe proPO band, the gels were incubated with trypsin (2 mg ml⁻¹ of CAC1 buffer) solution at 25 °C for 30 min, washed thrice with distilled water and then incubated with L-dopa (3 mg ml⁻¹ of CAC1 buffer) for 45 min. Gels were then washed three times with distilled water and stained with 0.1 % coomassie brilliant blue for detecting the protein bands.

2.12. Protein estimation

Protein concentration in HLS and fractions of column during gel filtration was estimated by the method of Lowry *et al* [12] using bovine serum albumin as standard.

2.13. Statistical analysis

Significance was determined with Student's *t*-test and differences between results were considered significant at $P < 0.05$.

3. RESULTS

The main biochemical and molecular properties of prophenoloxidase in haemocytes of *S. tranquebarica* are given below.

3.1. Presence of phenoloxidase activity

The spectrometric analysis in the present study revealed the oxidation of L-dopa by PO in HLS of crab, *S. tranquebarica* (Table 1). The activity of PO was found to be higher in HLS than in plasma and it needs a serine protease like trypsin to convert proPO to active PO. Thus, the result confirms the distribution of proPO in this crab is confined to haemocytes and haemocyte lysate can be used for determining its activity.

Table 1: PO activity from haemocyte lysate supernatant (HLS) and plasma of *Scylla tranquebarica* (mean \pm SD; n = 8).

Sample	PO activity (units min ⁻¹ mg ⁻¹ of protein)
HLS	134.18 \pm 3.48
Plasma	2.42 \pm 0.45

3.2. Substrate specificity, Km and pH determination for PO activity

To determine the PO specificity, various concentrations (0.5-30 mM) of L-dopa and phenol were used as substrates. Interestingly the results showed higher specificity for L-dopa than phenol. Table 2 shows the K_m (mM) and V_{max} (Δ Abs min⁻¹) based on Lineweaver-Burk model for the above substrates.

Table 2: K_m and maximum velocity of crab proPO with L-dopa and phenol as substrates.

Substrate(s)	K_m (mM)	V_{max} (Δ Abs min ⁻¹)
L-dopa	2.10	2.458
Phenol	4.03	0.003

Meanwhile, the enzyme was found to be inactive at pH 3.0 and 3.5, while above 6.0 the activity increased sharply reaching the maximum at pH 8.0 and thereafter it decreased (Fig 1).

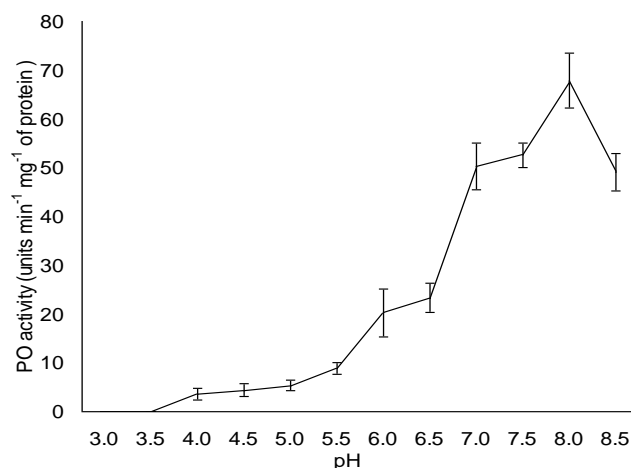


Fig. 1: Determination of optimum pH for PO activity. (mean \pm SD; n = 8).

3.3. Effect of exogenous elicitors

The results showed that PO activity in *S. tranquebarica* was enhanced significantly by pre-treatment with various species of Gram negative and Gram positive bacteria (Fig 2).

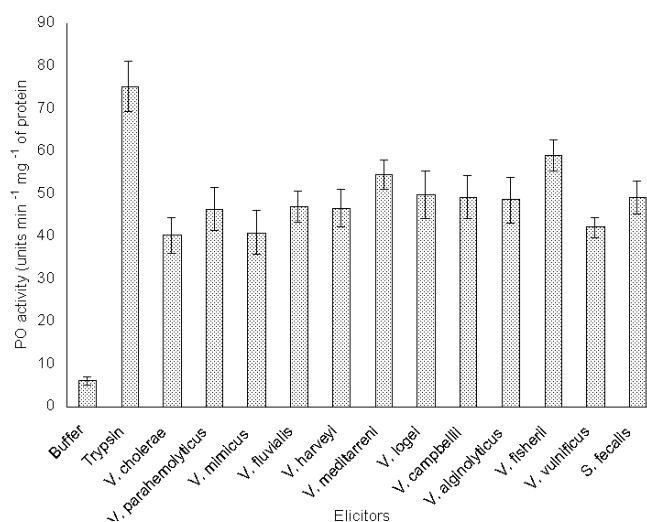


Fig. 2: Effect of exogenous elicitors on PO activity of haemocyte lysate from *Scylla tranquebarica*. (mean \pm SD; n = 6).

Among the *Vibrio* spp, *V. fisherii* (58.94 \pm 3.63 units) and *V. mediterranei* (54.50 \pm 3.22 units) elicit the highest PO activity while, *V. mimicus* (40.88 \pm 5.13 units) and *V. cholerae* (40.24 \pm 4.21 units) express the least activity.

Meanwhile, the Gram positive bacteria, *Streptococcus fecalis*, elicit phenoloxidase activity of 49.00 \pm 3.90 units. However, the stimulative effect of trypsin was found to be significantly ($P < 0.05$) higher than that by the different microbes used in the study. The presence of PO activity in buffer control could be due to formation of PO during preparation or handling.

3.4. Effect of inhibitors on PO activity

Significant ($P < 0.05$) reduction in PO activity of *S. tranquebarica* was observed in the presence of inhibitors. The average PO activity after addition of 10 mM of thiourea, sodium azide and EDTA was found to be 30.91 \pm 1.97, 48.37 \pm 7.00 and

50.47 ± 2.15 units, respectively. Meanwhile, in control, the PO was found to be 73.59 ± 6.35 units in the absence of inhibitors. The result implies that these chemicals can partially prevent the expression of the enzyme.

3.5. Effect of calcium and magnesium dependence of PO activity

The expression of PO depends on the presence of Ca²⁺ or Mg²⁺ ions (Fig 3). A concentration of either 20 mM CaCl₂ (46.27 ± 3.32 units) or 20 (53.92 ± 2.43 units) to 50 mM MgCl₂ (54.50 ± 2.71 units) added *in vitro* showed the highest PO activity. Decreased activity was observed in both below and above this level of divalent cations.

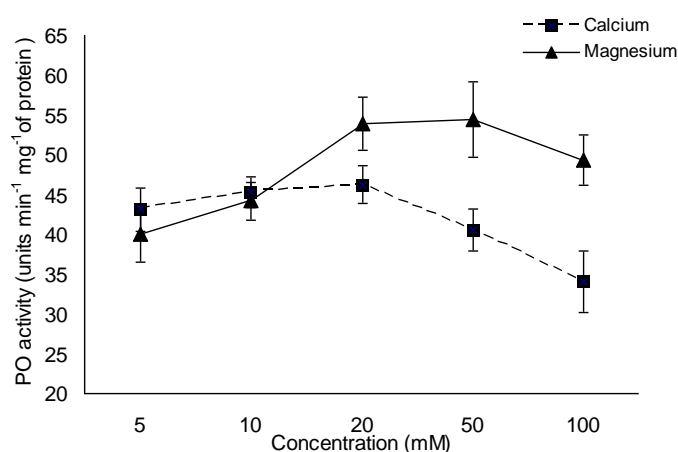


Fig. 3: Stimulative effect of calcium and magnesium ions on PO activity in HLS of *Scylla tranquebarica*. (mean ± SD; n = 8).

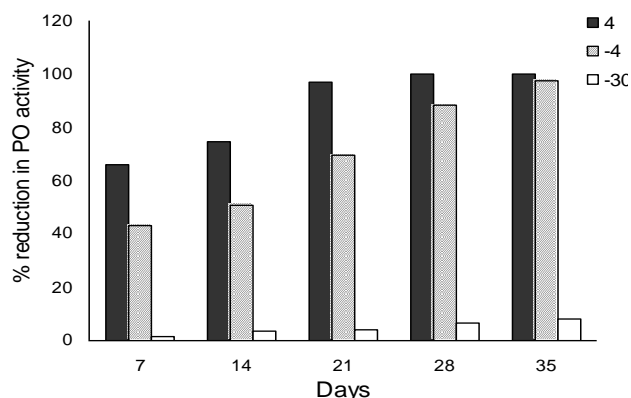


Fig. 4: Storage stability of proPO in HLS of crab, *Scylla tranquebarica*. (n = 6).

3.6. Storage stability

The stability of enzyme at three different temperatures (4, -4 and -30 °C) was evaluated at weekly intervals for 5 weeks. The reduction in enzyme activity was calculated as percentage and is shown in Fig 4. The proPO became inactivated after 3 weeks of storage at 4 °C, while it shows some activity even on 35th day when stored at -4 °C. The activity of this enzyme in HLS could preserve for a long time (35 days) on storage at -30 °C. Thus it can be concluded from the study that proPO is sensitive to storage conditions.

3.7. Gel filtration chromatography

The elution profile of haemocyte lysate of *S. tranquebarica* by Sephadex G200 gel is shown in Fig 5. The protein profile of eluates revealed first two prominent peaks followed by several minor peaks. Fraction number 6 to 22 showed significant quantity of protein and PO activity while fraction no 23 to 76 has no PO activity but contain varying levels of low protein concentration. Based on the above observations, the fractions containing highest PO activity were pooled and concentrated for use in polyacrylamide gel electrophoresis (PAGE).

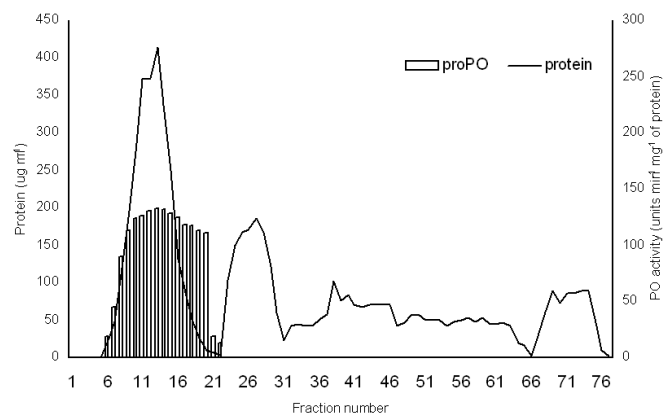


Fig. 5: Elution profile of haemocyte lysate by Sephadex G-200 column chromatography.

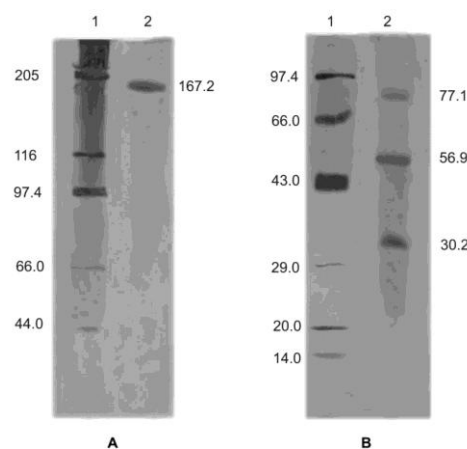


Fig. 6: Electrophoresis of the enzyme proPO from the haemocyte lysate of *Scylla tranquebarica*.

(A) Native PAGE of proPO purified by gel filtration chromatography. Lane 1, protein molecular weight markers; lane 2, proPO. (B) SDS-PAGE of proPO purified by gel filtration chromatography. Lane 1, protein molecular weight markers; lane 2, proPO.

To determine the molecular weight, the enzyme was subjected to native and SDS-PAGE. After running the native PAGE, the gel, treated with trypsin and L-dopa revealed a faint black patch and later it absorbs the coomassie blue stain and appears as a protein band. This protein is proPO and appears as a single prominent band having approximately 167.2 kDa (Fig 6A). At the same time, there was no observable dark patch or band after treating the SDS PAGE gel with trypsin and L-dopa. But after

staining, three bands having molecular weights of approximately 77.1, 56.9 and 30.2 kDa (Fig 6B) were visible.

4. DISCUSSION

Microbes and parasites can naturally enter into the arthropod through wounds or contamination of food. The response to this entry can often be visible as dark spots in the cuticle of the arthropods. This colour is caused by melanin, which is produced as a product of the proPO system in arthropods. The enzyme responsible for the melanin formation is phenoloxidase (PO), which catalyses the oxidation of phenols to quinines that subsequently polymerize into melanin. During the formation of melanin, toxic metabolites are formed with reported antimicrobial properties [4].

It is well documented that in arthropods, the zymogenic form, proPO is converted to PO through a proteolytic cleavage by a serine protease such as trypsin [13] and this activity has been demonstrated in haemocyte lysate of decapod crustaceans [14]. Similarly, the present study confirms the location of PO as proPO in haemocytes of *S. tranquebarica*.

S. tranquebarica PO showed highest specificity for L-dopa as substrate. In general, the enzyme-catalysed reactions proceed via the formation of enzyme substrate complex in which substrate binds non-covalently to the active site of the enzyme [15] and the use of L-dopa as substrate in detecting the PO activity was reported in many crustaceans. The K_m value for L-dopa in PO activity of this crab was found to be much close to the reports from *Penaeus californiensis* [7]. Moreover, PO of crustaceans has higher activity with diphenols and the result of the present study is comparable to the above reports from various invertebrates. The optimum pH for PO activity is 8.0 in *S. tranquebarica*, which was similar to brown shrimp, *P. californiensis* [7]. pH forms an important factor for the enzyme action because the ionization of amino acid residues in the active site of an enzyme depends on the optimum pH [13].

In our study using bacteria as elicitors, both Gram positive and Gram negative microbes stimulate the inactive proenzyme and convert to active PO. The proPO system is known to be triggered by the cell wall components of bacteria namely lipopolysaccharides and peptidoglycan of gram negative and gram positive bacteria and recognition of these microbial polysaccharides result in the stimulation of a serine protease (prophenoloxidase activating enzyme: ppAE) that cleaves proPO at a specific peptide bond near its amino terminus, which activates this enzyme from haemocytes [4, 16]. The highest level of PO activity was reached after incubating with trypsin and this could be due to the complete or near maximum active site formation for the substrate than that of the microbes. Meanwhile, sodium azide, thiourea and EDTA were able to inhibit the expression of PO of *S. tranquebarica* and this occurs when inhibitors combine specifically with the enzyme to reduce its ability to convert substrate into product [15]. In the present study, the optimum requirement of Ca^{2+} and Mg^{2+} were checked separately for PO activity and

revealed that either of these cations helps in PO expression of *S. tranquebarica*. Generally, the arthropod proPO system required a threshold concentration of calcium for proPO activation [16] and the optimum levels of these two divalent cations, either separately or in combination of both has been reported in several decapods [5,18]. Furthermore, the ionic concentration in the hemolymph of decapods varies with environmental conditions, such as temperature and salinity [19]. Combining this fact and the result of the present study, the quality of the pond water is critical in maintaining the optimal levels of these divalent ions in order to stimulate the proPO system and strengthen the defence mechanism of *S. tranquebarica*. Higher concentrations of both ions invariably suppress the PO activity. The molecular mass of proPO in *S. tranquebarica* was found to be 167.2 kDa (Fig 6A). Several earlier reports suggest a wide range of molecular mass for proPO in arthropods. It exists as a monomeric protein weighing 113.9 kDa in *P. californiensis* [7], a tetramer having 300 kDa in crayfish [20], 37 kDa in *Procambarus clarkii* [21] and 69.5 kDa in *Charybdis japonica* [22].

5. CONCLUSION

In conclusion, phenoloxidase exists as a proenzyme (proPO) in the haemocytes of *S. tranquebarica*. The enzyme shows the substrate specificity to L-dopa with optimum activity at pH 8.0. The activation responses towards polysaccharides containing microbes and inhibitory properties towards different chemicals *in vitro* resemble the proPO activation system of many related decapod crustaceans and also requires divalent cations like Ca^{2+} or Mg^{2+} in significant concentrations for its activity. The proenzyme can store at $-30^{\circ}C$ without losing its activity for a minimum period of 35 days. The proPO has a molecular mass of approximately 167.2 kDa on native PAGE, which was resolved into three bands with molecular mass of approximately 77.1, 56.9 and 30.2 kDa. Future studies like sequencing and molecular cloning can reveal the critical functional roles of this enzyme cascade in *S. tranquebarica*.

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