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IN VITRO SERUM PHENOLOXIDASE ACTIVITY IN THE HEMOLYMPH OF FRESHWATER CRAB *PARATELPHUSA JACQUEMONTII* (RATHBUN, 1905)

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Abstract: **The objective of the current study was to comprehend the immune response in freshwater crabs, *Paratelphusa jacquemontii*. The purpose of this study was to demonstrate serum phenoloxidase (PO) activity. Different phenolic substrates were used to test the PO enzymes substrate affinity, and DL-3,4-dihydroxyphenylalanine (DL-DOPA) demonstrated the enzyme's highest substrate affinity. When the enzyme activity of serum with various DL-DOPA concentrations was evaluated, it showed that the enzyme activity was highest at 5 mM of DL-DOPA concentration. When the DL-DOPA enzyme activity was examined at various time intervals, it demonstrated stable activity at 10 min incubation duration. When the impact of various buffer ionic strengths on the oxidation of DL-DOPA by serum was evaluated, greater enzyme activity was found at a concentration of 50 mM Tris-HCl. Studying the impact of pH and temperature on the oxidation of DL-DOPA by serum, pH 7.5 and 25 ˚C were shown to have steady action. 10 mM phenylthiourea (PTU) inhibited the serum PO activity while trypsin, sodium dodecyl sulphate (SDS), and non-self molecule laminarin activated it. We also discovered the substances that in serum with strong PO activity inhibited or activated the enzyme *in vitro*, indicating for the first time an immunological function.**

***Keywords: Paratelphusa jacquemontii*, innate immunity, phenoloxidase, inhibition and activation, enzyme activity.**

I. INTRODUCTION

Crabs constitute a significant portion of the freshwater ecosystem, where they are linked to different parts of the food webs in aquatic ecosystems. They are active predators and detritus feeders, occupying an intermediate position in both aquatic and terrestrial food webs (Maharajan *et al*., 2017). It is notable that crustaceans have been one of the most extensively studied groups of crustaceans, obviously due to their size, wide distribution in varying habitats, and potential as a source of food with good quality protein as well as their potential for intensive aquaculture (Denis *et al*., 2017a, b; Shanthi *et al*., 2021). The crab immune system, like that of other invertebrates, lacks an adaptive immune system and relies solely on its innate immunity against invading pathogens. Innate immunity is an ancient protective mechanism that appeared early in the evolution of metazoans and is divided into humoral and cellular responses (Denis *et al.*, 2017a, b).

The invertebrate immune system has a simple and primitive defense system consisting of cellular and humoral components (Fredrick & Ravichandran, 2012; Denis *et al*., 2017a, b; Sivakumar *et al.*, 2017; Tassanakajon *et al*., 2018; Huang & Ren, 2020; Huang *et al*., 2020; Sivakumar *et al*., 2020; Shanthi *et al*., 2021; Shanthi & Sivakumar, 2022; Sivakumar & Shanthi,

2022; Sivakumar *et al*., 2022; Sivakumar & Shanthi, 2023). In crustaceans, the cellular immune components primarily include certain fixed cells such as branchial podocytes, nephrocytes, etc., and circulating blood cells, or hemocytes (Smith & Ratcliffe, 1980; Johnson, 1987; Martin *et al.*, 1998; Denis *et al*., 2017a; Sivakumar *et al*., 2022).

The humoral immune components include a variety of soluble substances detectable in the plasma or sera, such as agglutinins (Maheswari *et al.*, 2002; Denis *et al*., 2015; Denis *et al*., 2017a; Mohanty *et al*., 2020; Sivakumar *et al*., 2020). In the prophenoloxidase (proPO) system, recognition of overseas material is thought to arise through popular molecules in the blood (hemolymph) of invertebrates. These set off activation of the prophenoloxidase activating system, and may also result in activation of different defense approaches (Söderhäll & Cerenius, 1998; Denis *et al*., 2017a; Sivakumar *et al*., 2017; Sivakumar & Shanthi, 2022, 2023), β-1,3 glucan and bacterial LPS-binding proteins (Hoffmann *et al*., 1999; Destoumiéux *et al.*, 2001; Lee & Söderhäll, 2002; Yu & Kanost, 2002), antibacterial proteins (Destoumiéux *et al.*, 1997), antimicrobial peptides and melanization (Tassanakajon *et al*., 2018) hemolytic system (Mercy & Ravindranath, 1994; Milochau *et al*., 1997; Lange & Magnadóttir, 2003; Zhang *et al*., 2009; Yan *et al*., 2011b; Marggraf *et al*., 2018), antifungal proteins (Iijime *et al*., 1993), as well as an array of cytotoxic molecules (Söderhäll *et al.*, 1986; Marggraf *et al*., 2018).

The copper chelating agent, phenylthiourea (PTU) is used as a common PO inhibitor. It was discovered that oxygen consumption was reduced in the presence of PTU for various phenol oxidations catalyzed by PO. Later, a series of thiourea derivatives were studied, and PTU changes were found to be the most effective melanization inhibitors (Ryazanova *et al*., 2012; Wright *et al*., 2012; Sivakumar *et al*., 2017; Sivakumar & Shanthi, 2022, 2023). This could be due to copper chelation via PTU, sodium diethyldithiocarbamate (DETC) and β-mercaptoethanol, as copper is essential for phenoloxidase activity. The specific inhibitors of PO activity tested were phenylthiourea, β-mercaptoethanol, DETC and tropolone (Wright *et al*., 2012; Denis *et al*., 2017a; Sivakumar *et al*., 2017; Sivakumar & Shanthi, 2022, 2023; Sivakumar *et al*., 2022).

Interestingly, recent report on the melanization cascade, where phenoloxidase is the terminal enzyme, appears to play a key role in the recognition of and protection against microbial infections in invertebrates. Right here, we show that phenoloxidase activity and melanization are critical for the immune defense in the direction of an exceptionally pathogenic bacterium (Liu *et al*., 2007). Phenoloxidase activity was found inside the hemocyte lysate supernatant (HLS) and plasma after their incubation with trypsin (Perdomo-Morales *et al*., 2007; Denis *et al*., 2017a; Sivakumar *et al*., 2017; Sivakumar *et al*., 2022; Sivakumar & Shanthi, 2023).

The PO activity includes the presence of low concentrations of the well-known detergent sodium dodecyl sulphate (SDS). The PO is observed in nearly all organisms, functioning as an initiator of melanin synthesis. Fatty acids, small antimicrobial peptides, phospholipids, alcohols, and detergents have all been identified as ability activators of PO activity in vivo (Baird *et al*., 2007). The phenoloxidase, a copper-containing enzyme, catalyzes the synthesis of melanin and is related to cellular immune responses such as phagocytosis, nodulation, and encapsulation. The processes of phagocytosis consist of lytic activity and exocytosis following degranulation (Denis *et al*., 2017a; Liu *et al*., 2020).

The SDS, a synthetic activator, appears to mimic the natural activation of PO, but the mode of this activation is not recognized. It's been suggested that SDS induces a conformational transition in PO, which results in a gap in the entrance to the lively website (Marques *et al*., 1995). The enzymatic activity of phenoloxidase is assayed continuously in the presence of SDS (Sivakumar *et al*., 2017). Comparable assay situations elicit phenoloxidase activity in another type 3 copper proteins, specifically hemocyanin, which typically functions as an oxygen provider. The character of the conformational modifications brought about in kind three copper proteins by means of the denaturant SDS is unknown (Decker *et al*., 2001; Baird *et al*., 2007).

Recent investigations have suggested that all known hemolymph phenoloxidases occur as precursors, proPO, and that these precursors are activated through a cascade of reactions triggered by minute amounts of microbial cell wall materials such as fungal cell wall components (β-1,3 glucan), peptidoglycans of the gram-positive bacteria cell wall (Yoshida & Ashida, 1986; Sivakumar *et al*., 2017; Shanthi *et al*., 2021) and lipopolysaccharides (Ratcliffe *et al*., 1984; Ashida & Yamazaki, 1990; Söderhäll, 1992; Sivakumar *et al*., 2017) and hence are claimed to be a part of the non-self-recognition system in invertebrates. Although all the steps of the cascade have not been elucidated, some vital features have emerged in arthropods, especially in crustaceans and insects (Ashida & Yamazaki, 1990; Söderhäll, 1992; Sivakumar *et al*., 2017).

A few activators were used, such as laminarin, zymosan, lipopolysaccharide (LPS) and trypsin this represented the spontaneous PO activity. This allowed the demonstration of true phenoloxidase activity and the technique of PO activation to be characterized (Hellio *et al*., 2007). The β-1,3-glucans in laminarin also served to spark off proPO activity as a non-self sugar molecule. Interestingly, the SDS triggered conformational changes and more advantageous PO activity than that of the proteolytic cleavage or via non-self stimulation (Denis *et al*., 2017a; Sivakumar *et al*., 2017).

Through *in vitro* activation of hemocyte, β-1,3-glucans such as laminarin or zymosan supernatant suppress hyphae-conditioned medium and show a decrease in the production of the cell-associated enzyme and phenoloxidase. *In vivo*, the impact of destruxins on β-1,3-glucan-brought about haemocytic aggregation (nodule formation) is assessed *in vitro*, and their effects on β-1,3-glucan precipitated prophenoloxidase-activation in living haemocytes and haemocyte lysate, on protein release and on haemocyte locomotory behaviour are observed (Huxham *et al*., 1989; Denis *et al*., 2017a; Sivakumar *et al*., 2022).

In the present study, inhibitors and activators of serum phenoloxidase activity have been analyzed and the effect of DL-DOPA in conjunction with some primary parameters is examined. This has a look at and presents an evaluation of the conformational modifications in hemocyanins associated with the addition of SDS (Baird *et al*., 2007). Some activators, such as the protease trypsin and the detergent SDS and β-1,3 glucan in laminarin, aid in the activation of proPO activity in *P. jacquemontii* and *Albunea symmysta* (Denis *et al*., 2017a; Shanthi *et al*., 2021). The present study, for the first time, aims to optimize conditions for serum PO activity and determine the effects of inhibition and activation on the phenoloxidase activity of the freshwater crab *P. jacquemontii*.

II. MATERIALS AND METHODS

Collection and maintenance of experimental crabs:

Adult intermoult individuals of the freshwater crab *Paratelphusa jacquemontii* were collected from the Thiruvallur District, Tamil Nadu, India. The crabs were maintained in the laboratory in tanks (0.5 × 0.5 m) with water to just immerse the crabs and being a tropical place the temperature remained moderate (27–30˚C) throughout the year. Crabs were acclimatized to laboratory conditions for two days before use.

Hemolymph collection and preparation of serum:

The exuded hemolymph from the excised dactyl of the intermoult crabs, of either sex, uninjured, weighing 50–55 g, was collected in a test tube kept on ice and centrifuged at 7000 ×g, 20 min at 4˚C to obtain the serum as supernatant. The serum used for protein estimation was immediately frozen at –20˚C until assayed. The protein content in serum was determined using bovine serum albumin as the standard following Bradford (1976). The serum was freshly prepared and used immediately for measuring PO activity. All chemicals during this and other assays were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Optimal conditions for serum PO activity:

Oxidation of phenolic substrates by serum:

We tested the oxidative activity of 0.1 ml serum by incubating with 0.9 ml of different phenolic substrate solutions (Tris-HCl 10 mM, pH 7.5; 5 mM-tyrosine, tyramine, L-DOPA, DL-DOPA, dopamine, catechol, hydroquinone, and pyrogallol) for 10 min at 25˚C. The colour developed was measured spectrophotometrically (UV-160A spectrophotometer, Shimadzu, Kyoto, Japan) at 300–700 nm against a reagent blank in which particular substrates were substituted for serum.

Effect of different concentration of DL-DOPA by serum:

The oxidative activity of serum to DL-DOPA was tested by incubating 0.1 ml of serum in 0.9 ml of DL-DOPA (Tris-HCl 10 mM, pH 7.5) with different concentrations of DL-DOPA (1.0–10.0 mM) for 10 min at 25˚C. The colour developed was measured spectrophotometrically at 480 nm against a reagent blank (DL-DOPA).

Effect of different time interval of DL-DOPA by serum:

We similarly measured color development way in all further tests using 0.1 ml of serum with 0.9 ml of 5 mM DL-DOPA (Tris-HCl 10 mM, pH 7.5) incubated for different time intervals for 1–60 min at 25˚C.

Effect of ionic strength on oxidation of DL-DOPA by serum:

The effect of ionic strength of the buffer on oxidation of 5 mM DL-DOPA by serum was assessed by incubating 0.1 ml of serum with 0.9 ml of DL-DOPA prepared in Tris-HCl buffer (pH 7.5) with different ionic strength (5–100 mM) at 25˚C.

Effect of pH on oxidation of DL-DOPA by serum:

The ability of oxidation of 5 mM DL-DOPA at different pH values was tested by incubating 0.1 ml of serum with 0.9 ml of substrate solution prepared in Tris-HCl buffer (50 mM) at pH 6.0–9.0 for 10 min at 25˚C.

Effect of temperature on oxidation of DL-DOPA by serum :

The effect of different temperatures was tested by incubating 0.1 ml of serum with 0.9 ml solutions of the substrate (5 mM DL-DOPA) prepared in 50 mM Tris-HCl (pH 7.5) buffer at temperatures of 10–80°C for 10 min.

Effect of inhibition on serum PO activity:

Phenylthiourea at various concentrations (1–12.5 mM) was prepared from which 0.1 ml was added to 0.1 ml of serum. PTU was substituted as a control with Tris-HCl buffer (50 mM, pH 7.5) and pre-incubated for 5 min at 25°C. The reaction mixtures from the control or experimental trials (0.2 ml) were incubated with 0.8 ml of DL-DOPA (5 mM) for 10 min at 25°C. The optical density of the control and experimental trials were measured spectrophotometrically at 480 nm.

Effect of activators on serum PO activity:

For experiment, 0.1 ml of serum was separately mixed with 0.1 ml of trypsin or SDS (1–12.5 $mg.ml^{-1}$), or laminarin (1–10 $mg.ml^{-1}$), and pre-incubated for 5 min at 25°C and then with 0.8 ml of substrate solution (5 Mm DL-DOPA) incubated for 10 min at 25°C. The colour developed was measured spectrophotometrically at 480 nm against the blank DL-DOPA.

Statistical analysis:

The data were expressed as mean ± SD of triplicate experimental trials from five determinations. The variation between trials and control were evaluated by SPSS/18 software (version 20; SPSS, New York) by one-way analysis of variance (ANOVA) at 0.05, 0.01 and 0.001probabilities ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

III. RESULTS

Serum PO activity with various substrates:

The serum separated from the hemolymph of freshwater crab *P. jacquemontii* showed the highest activity with diphenols DL-DOPA (480 nm) when compared to L-DOPA (500 nm), catechol (460 nm), and dopamine (490 nm), and polyphenols such as hydroquinone (520 nm) and pyrogallol (530 nm). In contrast, the monophenols, including tyramine and L-tyrosine failed to show any oxidation by the serum. Because the highest oxidative activity was obtained with DL-DOPA, this substrate was used to detect PO activity in all subsequent experiment (Fig. 1).

Effect of optimum substrate on DL-DOPA:

Serum PO activity increased with 1.0–10 mM concentrations of DL-DOPA. Of the tested concentrations, 5 mM was taken as the optimal concentration and was used for further investigation (Fig. 2).

Effect of optimum time interval:

PO activity was also tested by DL-DOPA at various time intervals (1–60 min). The highest PO activity was found at 60 min, but we are taken the 10 min, which was determined as the optimal incubation time (Fig. 3).

Effect of optimum ionic strength:

PO activity was also tested with Tris-HCl buffer (pH 7.5) of different ionic strengths (5–100 mM) and the highest activity was observed at 50 mM. This ionic strength was used as buffer concentration for further investigation (Fig. 4).

Effect of optimum pH:

Similarly, PO activity was tested with DL-DOPA of different pH values (6.0–9.0) of Tris-HCl buffer (50 mM). The highest PO activity was observed at pH 7.5, decreasing above and below a pH of 7.5 (Fig. 5).

Effect of optimum temperature:

Serum samples were incubated for 10 min at different temperatures ranging 10–80°C. Stable and peak PO activity was observed at 25°C, so this temperature was considered optimal (Fig. 6). In summary, the optimal conditions for measuring serum PO activity on DL-DOPA (5 mM) were 50 mM Tris-HCl, pH 7.5 at 25°C for 10 min at 480 nm (Fig. 6).

Effect of inhibition on serum phenoloxidase activity:

The serum PO activity inhibition was tested with different concentrations of PTU and the PO activity was found to be highest inhibited with 10 and 12.5 mM concentration than that of other concentrations. These results clearly suggest that the optimum concentration chosen for inhibiting the PO activity is 10 mM of PTU ($^{***}p< 0.001$; Fig. 7).

Effect of activators on serum phenoloxidase activity:

The serum PO activity was tested with different concentrations of trypsin, SDS or laminarin and the PO activity was found to be higher with 7.5, 10 or 2.5 mg.ml^{-1} concentration than that of other concentrations. These results clearly suggest that the optimum concentration for activating PO activity is 7.5, 10 or 2.5 mg.ml^{-1} of trypsin, SDS or laminarin ($^{*}p< 0.05$, $^{**}p< 0.01$, $^{*}p< 0.05$; Fig. 8A-C).

IV. DISCUSSION

In the present study, we chose to determine PO activity in the serum of *P. jacquemontii* to analyze the optimal conditions of various parameters such as substrate wavelength, specificity, concentrations, time intervals, ionic strength, pH and temperature for the enzyme activity. Substrate specificity for PO activity in the serum of *P. Jacquemontii* appeared for *o*-diphenols such as DL-DOPA, L-DOPA, dopamine and Catechol. It was observed that the substrate specificity for serum PO activity was high with DL-DOPA when compared to other substrates that were used. Moreover, PO from crustaceans has higher activity with diphenols (Sabu *et al*., 2016; Shanthi *et al*., 2021). This result was similar to the earlier findings of diphenoloxidase activity in *Panulirus argus* and *P. cygnus* (Chen *et al*., 1991).

The entire study of phenoloxidase activity was measured spectrophotometrically at 480 nm by recording the formation of dopachrome produced from DL-DOPA (Shanthi *et al*., 2021). The PO assays were regularly measured spectrophotometrically in the similar range of 400-500 nm absorbance, such as 475 nm in *Panulirus argus* (Ferrer *et al*., 1989; Chen *et al*., 1991), *P. cygnus* (Chen *et al*., 1991), *Callinectes sapidus* (Tanner *et al*., 2006), *Cancer magister* (Green, 2007), at 490 nm in *Macrobrachium rosenbergii* (Cheng *et al*., 2002), *Scylla tranquebarica* (Sabu *et al*., 2016) and *P. jacquemontii* (Denis *et al*., 2017a).

Every enzymatic reaction occurs under a particular biochemical condition hence certain biochemical assays were made to understand the optimum conditions for serum PO activity. The study of serum PO activity with different concentrations of DL-DOPA resulted in high activity at 5 mM concentration, which is similar to other crustacean species such as *Penaeus japonicus* (Zhao *et al*., 1997) and *A. symmysta* (Shanthi *et al*., 2021).

For better understanding, PO activity was tested with various concentrations of Tris-HCl buffer. The oxidation activity of serum PO was high at a 50 mM concentration. The results of the study were compared to the earlier findings of hemocyanin PO assays in *M. rosenbergii* (Sivakumar *et al*., 2017) and *P. argus* (Perdomo-Morales *et al*., 2007), where they used the same 50 mM Tris buffer concentration hence the entire study was carried out with this buffer concentration.

In the present study, the oxidation of substrate was tested at various time intervals from 1 to 60 min at an absorbance of 480 nm to assess the ability of the serum PO to generate dopachrome and the highest activity was observed at 10 min of incubation, which is similar to the PO oxidation of *Penaeus vannamei* (García-Carreño, 2008), *M. rosenbergii* (Cheng *et al*., 2002) and *A. symmysta* (Shanthi *et al*., 2021) but varies from other crustacean species *Penaeus monodon* (Sung *et al*., 1998), *Penaeus paulensis* (Perazzolo & Barracco, 1997) having highest activity in 5 min of incubation and *S. tranquebarica* (Sabu *et al*., 2016) having higher oxidation in 20 min of incubation.

The pH forms an important factor for the enzyme action and it has been reported that the optimum pH is 8.0 for endogenously activated PO using DL-DOPA as a substrate they also noted that the PO activity was unstable below pH 4.0 and had a pH stability range of 6.0 –10.0 (Ferrer *et al*., 1989). In the present study, the oxidation activity of serum PO was stable at pH 7.5, which is similar to the proPO activity of white shrimp, *Penaeus setiferus* (Simpson *et al*., 1987), white leg shrimp, *P. vannamei* (García-Carreño *et al*., 2008) and anomuran crab, *A. symmysta* (Shanthi *et al*., 2021). It has also been reported that the activity of *Callinectes sapidus* PO against the *o*-diphenol substrate DL-DOPA increased with pH over the physiologically relevant range of pH 6.6–7.8 (Tanner *et al*., 2006). In addition, studies on Dungeness crab and Cancer magister revealed that pH levels lower than the optimum range suppressed PO activity (Green *et al*., 2007). In general, we observed that when the pH is either lowered below 6.0 or increased above 8.0, the serum PO activity gets reduced.

The changes in environmental conditions in the animal's natural habitat might cause differences in the optimal thermostability of the enzymes. In this study, we observed that the effect of temperature change affected serum PO activity. Most proPO enzymes are heat labile a short exposure of the enzyme to temperatures between 70–90˚C causes a partial or total irreversible denaturation (Chen *et al*., 1991). The oxidation of serum PO activity of *P. jacquemontii* was highly stable at 25˚C similar to the previous studies on serum PO activity of *Callinectes sapidus* (Tanner *et al*., 2006), *S. tranquebarica* (Sabu *et al*., 2016) and the anomuran crab, *A. symmysta* (Shanthi *et al*., 2021).

The gradual increase in temperature has been reported to decrease the serum PO activity because the serum gets denatured, like in shrimp (*Litopenaeus vannamei*) transferred to 32°C showed a significant decrease in PO activity (Cheng *et al*., 2005) and in brown shrimp (*Penaeus californiensis*) and crab (*Carcinus aestuarii*) exposure to increased temperature affected the prophenoloxidase system (Vargas-Albores *et al*., 1998; Matozzo *et al*., 2011). The enzyme activity decreased sharply at temperatures above 50°C where approximately 95% loss in the enzyme activity was detected at 80°C. In most of the crustaceans, this condition prevails, where a short exposure to high temperatures causes denaturation. The enzymes thermal stability is considered an important feature, making its use possible in numerous biotechnological processes that require a wide range of temperatures (Maalej *et al*., 2021).

In most of the investigated crustacean species, the enzyme precursor proPO was observed to be enclosed almost solely in their blood cells. However, there have been a few reports on a few low levels of enzyme activity within the plasma. The utilization of serum to detect proPO for aquaculture appears to be an alternative source for obtaining the proPO activating system for quick and preliminary analyses. This could be of interest for the monitoring of the freshwater crab immune status in cultivations since serum samples are much more readily and easily obtained (Perazzolo & Barracco, 1997; Denis *et al*., 2017a; Shanthi *et al*., 2021).

In the present study, inhibitors and activators of serum phenoloxidase activity were analyzed using the freshwater crab *P. jacquemontii*. The inhibitor used was PTU, a well-known and widely used inhibition of phenoloxidase. However, the mechanism of its action is not quite clear. The PO activity was found to be 98% inhibited at a 10 mM concentration compared to other concentrations, thus the optimum concentration for inhibiting the PO activity is 10 mM of PTU. The interaction of PTU with the enzyme inhibits the melanization process, and the inhibition may be due to copper chelation by PTU (Denis *et al*., 2017a; Sivakumar *et al*., 2017; Shanthi *et al*., 2021; Sivakumar & Shanthi, 2022; Sivakumar *et al*., 2022; Sivakumar & Shanthi, 2023).

The present study used the interesting activators trypsin, SDS, and laminarin. The proPO is converted to PO through proteolytic cleavage by trypsin (Cerenius & Söderhäll, 2004; Lee *et al*., 2004), and the phenoloxidase activity can be induced by SDS and laminarin. The activators, such as proteases like trypsin, detergents like SDS and β-1,3-glucan binding protein in laminarin, activated proPO activity in freshwater crab *P. jacquemontii* (Denis *et al*., 2017a), freshwater prawn *M. rosenbergii* (Sivakumar *et al*., 2017, 2022), and anomuran crab *A. symmysta* (Shanthi *et al*., 2021). Several components of the proPO activating system have been structurally determined in recent years, and a number of them have been attributed with new and important defence features. However, much remains to be learned about both the biochemical mechanism of activation of system constituent elements and their role in immune defence (Söderhäll & Cerenius, 1998).

In the present study, we conclude that the immunological function of phenoloxidase observed in the serum of the freshwater crab *P. jacquemontii* appears to enhance resistance against various diseases through the investigation, inhibition and activation of PO activity. This clearly indicates the significance of both cellular and humoral immune components in boosting the immune response. This result provides confirmation that, for the first time, the serum of *P. jacquemontii* is an effective immune system with the ability to enzymatically function as a phenoloxidase, and the immune response mechanism of hemocyanin in the hemolymph of *P. jacquemontii* requires further elucidation.

REFERENCES

[1] Ashida, M. & Yamazaki, I.H. (1990). Biochemistry of the phenoloxidase system in insects: with special reference to its activation. In Molting and Metamorphosis, (Edited by Ohnishi, E. & lshizaki, H.), (pp. 239–265), *Japan Science Society Press*, Tokyo/Springer. Berlin.

[2] Baird, S., Kelly, S.M., Price, N.C., Jaenicke, E., Meesters, C., Nillius, D., Decker, H. & Nairn, J. 2007. Hemocyanin conformational changes associated with SDS-induced phenol oxidase activation. *Biochimica et Biophysica Acta*, 1774, 1380–1394.

[3] Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, 72, 248–254.

[4] Cerenius, L. & Söderhäll, K. (2004). The prophenoloxidase-activating system in invertebrates. *Immunological Reviews*, 198, 116–126.

[5] Chen, J.S., Rolle, R.S., Marshall, M.R. & Wei, C.I. (1991). Comparison of phenoloxidase activity from Florida spiny lobster and Western Australian lobster. *Journal of Food Science*, 56, 154–157.

[6] Cheng, W., Liu, C.H., Hsu, J.P. & Chen, J.C. (2002). Effect of hypoxia on the immune response of giant freshwater prawn *Macrobrachium rosenbergii* and its susceptibility to pathogen *Enterococcus*. *Fish & Shellfish Immunology*, 13, 351–365.

[7] Cheng, W., Wang, L.U., & Chen, J.C. (2005). Effect of water temperature on the immune response of white shrimp *Litopenaeus vannamei* to *Vibrio alginolyticus*. *Aquaculture*, 250, 592–601.

[8] Decker, H., Ryan, M., Jaenicke, E. & Terwilliger, N. (2001). SDS-induced phenoloxidase activity of hemocyanins from *Limulus polyphemus*, *Eurypelma californicum*, and *Cancer magister*. *Journal of Biological Chemistry*, 276, 17796–17799.

[9] Denis, M., Karthigayani, T., Sivakumar, M.R. & Arumugam, M. (2015). Opsonic function of sialic acid specific lectin in freshwater crab *Paratelphusa jacquemontii*. *Springer Plus*, 4, 1–15.

[10] Denis, M., Sivakumar, M.R., Tamilarasan, K., Karthigayani, T., Prabu, M., Bhuvaneswari, S.D., & Arumugam, M. (2017a). Activation of phenoloxidase activity by humoral lectin in hemocytes of freshwater crab *Paratelphusa jacquemontii*. *International Journal of Biological Macromolecules*, 97, 258–263.

[11] Denis, M., Sivakumar, M.R., Karthigayani, T. & Arumugam, M. (2017b). Immune response of anti-lectin Pjlec antibody in freshwater crab *Paratelphusa jacquemontii*. *International Journal of Biological Macromolecules*, 104, 1212–1222.

[12] Destoumiéux, D., Bulet, P., Loew, D., Dorsselaer, A.V., Rodriguez, J. & Bachère, E. (1997). Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). *Journal of Biological Chemistry*, 27, 28398–28406.

[13] Destoumiéux-Garzón, D., Saulnier, D., Garnier, J., Jouffrey, C., Bulet P. & Bachère. E. (2001). Crustacean immunity. Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge. *Journal of Biological Chemistry*, 276, 47070–47077.

[14] Ferrer, O.J., Koburger, J.A., Otwell, W.S., Gleeson, R.A., Simpson, B.K. & Marshall, M.R. (1989). Phenoloxidase from the cuticle of Florida spiny lobster (*Panulirus argus*): Mode of activation and characterization. *Journal of Food Science*, 54, 63–67.

[15] Fredrick, W.S. & Ravichandran, S. (2012). Hemolymph proteins in marine crustaceans. *Asian Pacific Journal of Tropical Biomedicine*, 2, 496–502.

[16] García-Carreño, F.L., Cota, K. & Navarrete Del Toro, M.A. (2008). Phenoloxidase activity of hemocyanin in whiteleg shrimp *Penaeus vannamei*: conversion, characterization of catalytic properties, and role in postmortem melanosis. *Journal of Agricultural and Food Chemistry*, 56, 6454–6459.

[17] Green, S. (2007). The Effects of pH on phenoloxidase activity in the Brachyuran crab, *Cancer magister*. *Oregon Institute of Marine Biology*, pp1–14.

[18] Hellio, C., Bado-Nilles, A., Gagnaire, B., Renault, T. (&) Thomas-Guyon, H. (2007). Demonstration of a true phenoloxidase activity and activation of a proPO cascade in Pacific oyster, *Crassostrea gigas* (Thunberg) *in vitro*. *Fish & shellfish immunology*, 22, 433–440.

[19] Hernández-López, J., Gollas-Galván, T. & Vargas-Albores, F. (1996). Activation of the prophenoloxidase system of the brown shrimp (*Penaeus californiensis*, Holmes). *Comparative Biochemistry & Physiology C*, 113, 61–66.

[20] Hoffmann, J.A., Kafatos, F.C., Jr. Janeway, C.A. & Ezekowitz, R.A.B. (1999). Phylogenetic perspectives in innate immunity. *Science*, 284, 1313–1318.

[21] Huang, Y. & Ren, Q. (2020). Research progress in innate immunity of freshwater crustaceans. *Developmental & Comparative Immunology*, 104, 103569.

[22] Huang, Z., Aweya, J.J., Zhu, C., Tran, N.T., Hong, Y., Li, S., Yao, D. & Zhang, Y. (2020). Modulation of crustacean innate immune response by amino acids and their metabolites: Inferences from other species. *Frontiers in Immunology*, 11, 574721.

[23] Huxham, I.M., Lackie, A.M. & Mc Corkindale, N.J. (1989). Inhibitory effects of cyclodepsipeptides, destruxins, from the fungus *Metarhizium anisopliae*, on cellular immunity in insects. *Journal of Insect Physiology*, 35, 97–105.

[24] Iijima, R., Kurata, S. & Shunji, N. (1993). Purification, characterization, and cDNA cloning of an antifungal protein from the hemolymph of *Surcophugu peregrinu* (Flesh fly) larvae. *Journal of Biological Chemistry*, 268, 12055–12061.

[25] Johnson, P.T. (1987). A review of fixed phagocytic and pinocytotic cells of decapod crustaceans, with remarks on hemocytes. *Developmental & Comparative Immunology*, 11, 679–704.

[26] Lange, S. & Magnadóttir, B. (2003). Spontaneous haemolytic activity of Atlantic halibut (*Hippoglossus hippoglossus* L.) and sea bass (*Dicentrarchus labrax*) serum. *Comparative Biochemistry & Physiology B*, 136, 99–106.

[27] Lee, S.Y. & Söderhäll, K. (2002). Early events in crustacean innate immunity. *Fish & Shellfish Immunology*, 12, 421–437.

[28] Lee, S.Y., Lee, B.L. & Söderhäll, K. (2004). Processing of crayfish hemocyanin subunits into phenoloxidase. *Biochemical and Biophysical Research Communications*, 322, 490–496.

[29] Liu, H., Jiravanichpaisal, P., Cerenius, L., Lee, B.L., Söderhäll, I. & Söderhäll, K. (2007). Phenoloxidase is an important component of the defense against *Aeromonas hydrophila* infection in a crustacean, *Pacifastacus leniusculus*. *Journal of Biological Chemistry*, 282, 33593–33598.

[30] Liu, S., Zheng, S.C., Li, Y.L., Li, J. & Liu, H.P. (2020). Hemocyte-mediated phagocytosis in crustaceans. *Frontiers Immunology*, 11, 268.

[31] Maalej, H., Maalej, A., Affes, S., Hmidet, N. & Nasri, M. (2021). A novel digestive α-amylase from blue crab (*Portunus segnis)* viscera: purification, biochemical characterization and application for the improvement of antioxidant potential of oat flour. *International Journal of Molecular Sciences*, 22, 1070.

[32] Maharajan, A., Shanmugavel, K. & Parurukmani, P.S. (2012). Biochemical changes in haemolymph of fresh water crab, *Paratelphusa jacquemontii* (Rathbun) exposed to copper. *International Journal of Basic & Applied Sciences*, 1, 421–428.

[33] Maheswari, R., Mullainadhan, P. & Arumugam, M. 2002. Isolation and characterization of an acetyl group-recognizing agglutinin from the serum of the Indian white shrimp *Fenneropenaeus indicus*. *Archives of Biochemistry and Biophysics*, 402, 65–76.

[34] Marggraf, M.B., Panteleev, P.V., Emelianova, A.A., Sorokin, M.I., Bolosov, I.A., Buzdin, A.A., Kuzmin, D.V. & Ovchinnikova, T.V. (2018). Cytotoxic potential of the novel horseshoe crab peptide polyphemusin III. *Marine Drugs*, 16, 466.

[35] Marques L., Fleuriet, A. & Macheix, J.J. (1995). Characterization of multiple forms of polyphenol oxidase from apple fruit. *Plant Physiology and Biochemistry*, 33, 193–200.

[36] Martin, G.G., Kay, J., Poole, D. & Poole, C. (1998). *In vitro* nodule formation in the ridgeback prawn, *Sicyonia ingentis*, and the American lobster, *Homarus americanus*. *Invertebrate Biology*. 117, 155–168.

[37] Matozzo, V., Gallo, C. & Marin, M.G. (2011). Effects of temperature on cellular and biochemical parameters in the crab *Carcinus aestuarii* (Crustacea, Decapoda). *Marine Environmental Research*, 71, 351–356.

[38] Mercy, Sr. P.D. & Ravindranath, M.H. (1994). Hemolysis and clearance of erythrocytes in *Scylla serrata* are related to the agglutination by native sialic acid-specific lectin. *Comparative Biochemistry & Physiology A*, 109, 1075–1083.

[39] Milochau, A., Lassègues, M. & Valembois, P. 1997. Purification, characterization and activities of two hemolytic and antibacterial proteins from coelomic fluid of the annelid *Eisenia fetida Andrei*. *Biochimica et Biophysica Acta*, 1337, 123–132.

[40] Mohanty, J., Sahoo, S., Badhe, M.R,, Pillai, B.R., Sahoo, P.K., Suryawanshi, A.R. & Patnaik, B.B. (2020). Lectin like activity of hemocyanin in freshwater prawn *Macrobrachium rosenbergii*. *The Protein Journal*, 39, 358–365.

[41] Perazzolo, L.M. & Barracco, M.A. (1997). The prophenoloxidase activating system of the shrimp *Penaeus paulensis* and associated factors. *Developmental & Comparative Immunology*, 21, 385–395.

[42] Perdomo-Morales, R., Montero-Alejo, V., Perera, E., Pardo-Ruiz, Z. & Alonso-Jimenez, E. (2007). Phenoloxidase activity in the hemolymph of the spiny lobster *Panulirus argus*. *Fish & Shellfish Immunology*, 23, 1187–1195.

[43] Ratcliffe, N.A., Leonard, C. & Rowley, A.F. (1984). Prophenoloxidase activation: Nonself recognition and cell cooperation in insect immunity. *Science*, 226, 557–559.

[44] Ryazanova, A.D., Alekseev, A.A. & Slepneva, I.A. (2012). The phenylthiourea is a competitive inhibitor of the enzymatic oxidation of DOPA by phenoloxidase. *Journal of Enzyme Inhibition & Medicinal Chemistry*, 27, 78–83.

[45] Sabu, A.S., Jithendran, K.P. & Gopal, C. (2016). Properties of phenoloxidase from haemocytes of *Scylla tranquebarica*. *Journal of Applied Biology & Biotechnology*, 4, 47–52.

[46] Shanthi, S., Sivakumar, M.R. & Rayvathy, B. (2021). Serum phenoloxidase activity in the hemolymph of the anomuran crab *Albunea symmysta* (Linnaeus, 1758) (Decapoda: Anomura: Albuneidae). *The Journal of Crustacean Biology*, 41, ruab003.

[47] Shanthi, S. & Sivakumar, M.R. (2022). Serum phenoloxidase activity in the hemolymph of giant freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879). *International Journal of Zoology & Animal Biology*, 5, 000419.

[48] Simpson, B.K., Marshall, M.R. & Otwell, W.S. (1987). Phenol oxidase from shrimp (*Penaeus setiferus*): purification and some properties. *Journal of Agricultural & Food Chemistry*, 35, 918–921.

[49] Sivakumar, M.R., Denis, M., Shanthi, S. & Arumugam, M. (2017). Phenoloxidase activity in humoral plasma, hemocyanin and hemocyanin separated proteins of the giant freshwater prawn *Macrobrachium rosenbergii*. *International Journal of Biological Macromolecules*, 102, 977–985.

[50] Sivakumar, M.R., Denis, M., Shanthi, S. & Arumugam, M. (2020). Agglutination of plasma, hemocyanin, and separated hemocyanin from the hemolymph of the freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879) (Decapoda: Caridea: Palaemonidae). *The Journal of Crustacean Biology*, 40, 309–315.

[51] Sivakumar, M.R. & Shanthi, R. (2022). Plasma and hemocyanin phenoloxidase derived from the hemolymph of giant freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879). *Crustacea-Nano, Micro and Macro Studies*, pp. 1–20.

[52] Sivakumar, M.R., Abisha Adline, F. & Shanthi, R. (2022). Hemocytes lysate supernatant derived phenoloxidase activity in the hemolymph of giant freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879). *International Journal of Zoology & Animal Biology*, 5, 000403.

[53] Sivakumar, M.R. & Shanthi, R. (2023). Inhibition and activation of humoral phenoloxidase and their effect on divalent cations in the hemolymph of freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879). *International Journal of Zoology & Animal Biology*, 6, 000540.

[54] Smith, V.J. & Ratcliffe, N.A. (1980). Host defence reactions of the shore crab, *Carcinus maenas* (L.); clearance and distribution of injected particles. *Journal of the Marine Biological Association of the United Kingdom*, 60, 89–102.

[55] Söderhäll, K., Smith, V.J. & Johansson, M.W. (1986). Exocytosis and uptake of bacteria by isolated haemocyte populations of two crustaceans: evidence for cellular co-operation in the defence reactions of arthropods. *Cell & Tissue Research*, 245, 43–49.

[56] Söderhäll, K. (199)2. Biochemical and molecular aspects of cellular communication in arthropods. *Italian Journal of Zoology*, 59, 141–151.

[57] Söderhäll, K. & Cerenius, L. (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. *Current Opinion in Immunology*, 10, 23–28.

[58] Sung, H.H., Chang, H.J., Her, C.H., Chang, J.C. & Song, Y.L. (1998). Phenoloxidase activity of hemocytes derived from *Penaeus monodon* and *Macrobrachium rosenbergii*. *Journal of Invertebrate Pathology*, 71, 26–33.

[59] Tanner, C.A., Burnett, L.E. & Burnett, K.G. (2006). The effects of hypoxia and pH on phenoloxidase activity in the Atlantic blue crab, *Callinectes sapidus*. *Comparative Biochemistry & Physiology A*, 144, 218–223.

[60] Tassanakajon, A., Rimphanichyakit, V., Visetnan, S., Amparyup, P., Somboonwiwat, K., Charoensapsri, W. & Tang, S. (2018). Shrimp humoral responses against pathogens: Antimicrobial peptides and melanization. *Developmental & Comparative Immunology*, 80, 81–93.

[61] Vargas Albores, F., Hinojosa-Baltazar, P., Portillo-Clark, G. & Magallon-Barajas, F. (1998). Influence of temperature and salinity on the yellow leg shrimp, *Penaeus californiensis* Holmes, prophenoloxidase system. *Aquaculture Research*, 29, 549–553.

[62] Wright, J., McCaskill-Clark, W., Cain, J.A., Patterson, A., Coates, C.J. & Nairn, J. (2012). Effects of known phenoloxidase inhibitors on hemocyanin-derived phenoloxidase from *Limulus polyphemus*. *Comparative Biochemistry & Physiology B*, 163, 303–308.

[63] Yan, F., Qiao, J., Zhang, Y., Zhou, N., Liu, Y., Guo, L., Li, Y. & Chen, J. (2011). Hemolytic properties of hemocyanin from mud crab *Scylla serrata*. *Journal of Shellfish Research*, 30, 957–962.

[64] Yoshida, H. & Ashida, M. (1986). Microbial activation of two serine enzymes and prophenoloxidase in the plasma fraction of hemolymph of the silkworm, *Bombyx mori*. *Insect Biochemistry*, 16, 539–545.

[65] Yu, X.Q. & Kanost, M.R. (2002). Binding of hemolin to microbial lipopolysaccharide and lipoteichoic acid: an immunoglobulin superfamily member from insects as a pattern recognition receptor. *European Journal of Biochemistry*, 269, 1827–1834.

[66] Zhang, Y., Yan, F., Hu, Z., Zhao, X., Min, S., Du, Z., Zhao, S., Ye, X. & Li, Y. (2009). Hemocyanin from shrimp *Litopenaeus vannamei* shows hemolytic activity. *Fish & Shellfish Immunology*, 27, 330–335.

[67] Zhao, J., Qi, X.Y., You, Y.M., Wang, J.X. & Zhou, E.G. (1997). Study on some characteristics of phenoloxidase from Japanese prawn, *Penaeus japonicus*. *Journal of Shanghai Fisheries University*, 6, 157–65.

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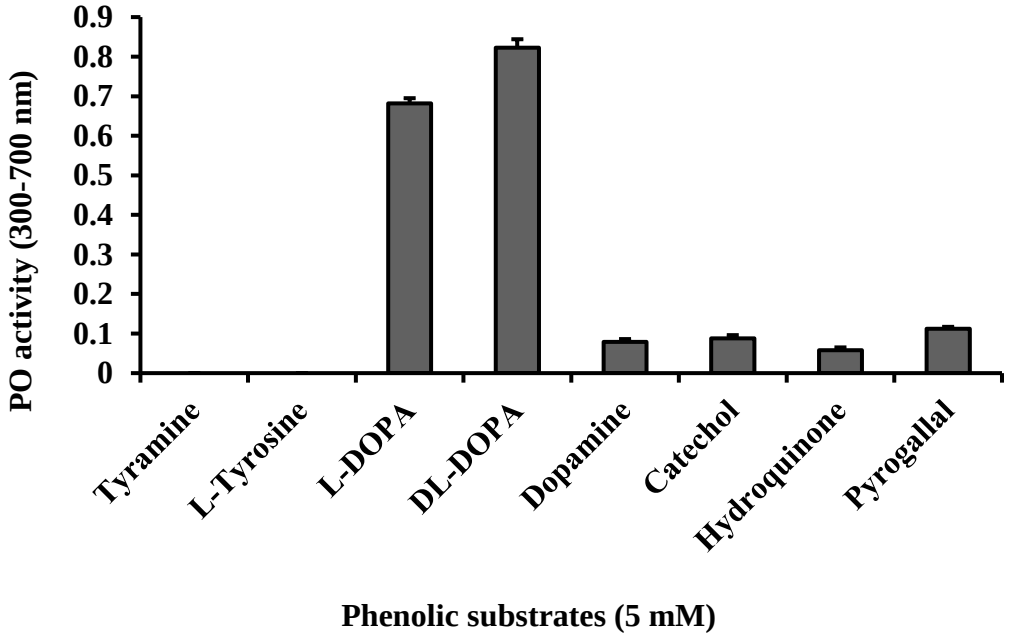

Fig 1: Phenoloxidase activity in the serum of *Paratelphusa jacquemontii* on different phenolic substrates (5 mM) in Tris-HCl buffer (Tris 10 mM, pH 7.5) incubated at 25˚C for 10 min and absorbance at 300–700 nm

The PO activity in optical density obtained at absorbance maxima of respective substrates. Data represent mean of triplicate repeats of five determinations (mean ± SD) in the same way in all further experiments.

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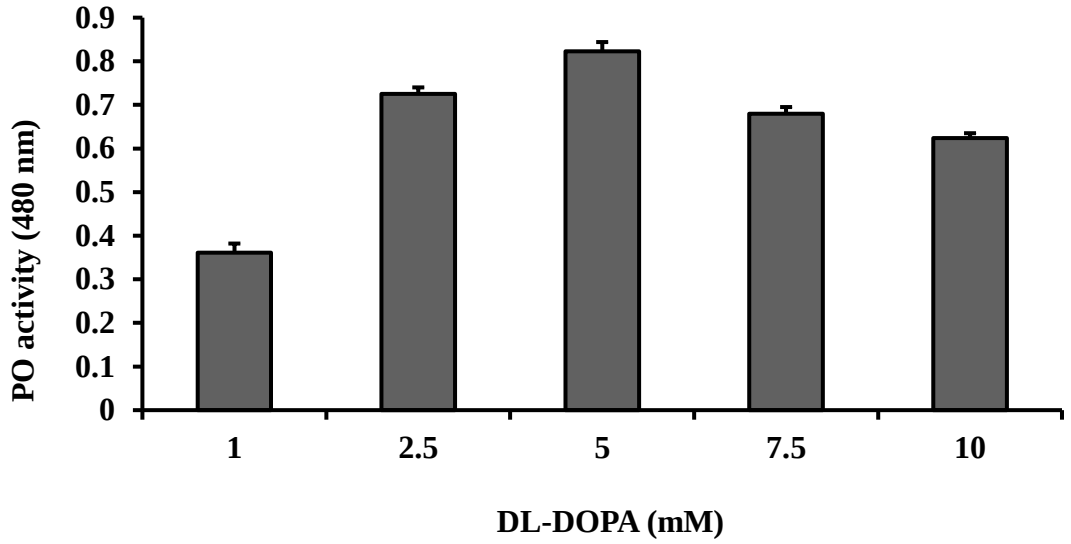

Fig 2: Effect of different concentrations of DL-DOPA in PO activity of the serum of *P. jacquemontii*

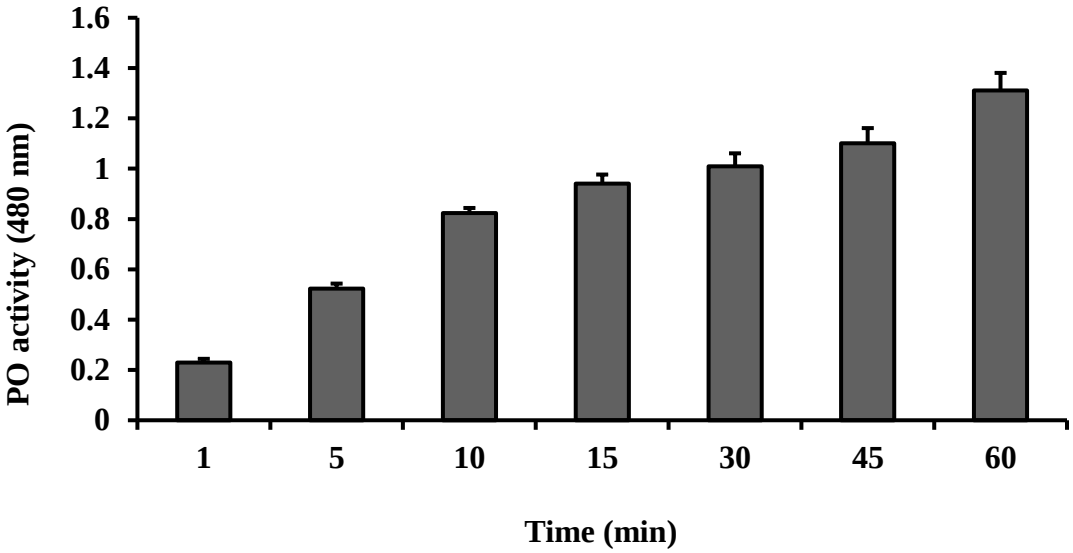

Fig 3: Effect of different time interval on oxidation of DL-DOPA in PO activity of the serum of *P. jacquemontii*

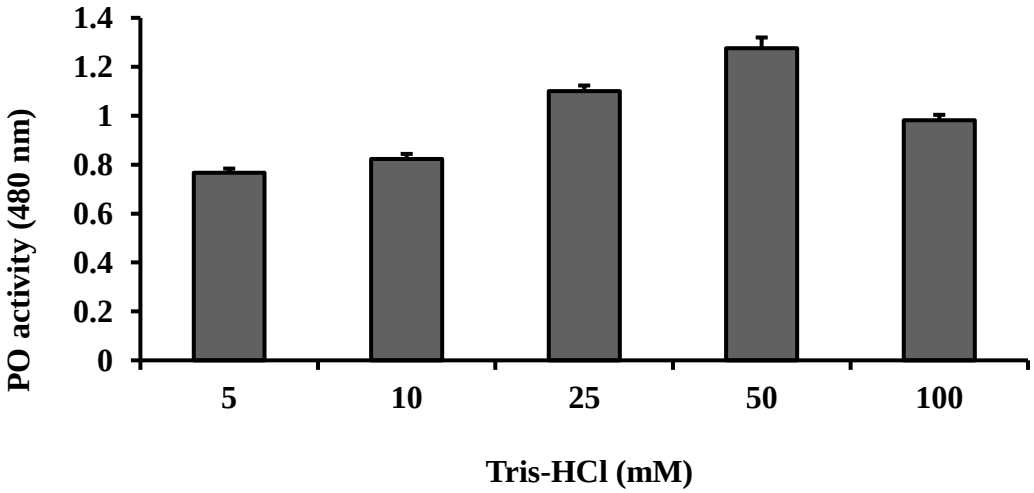

Fig 4: Effect of optimum ionic strength on oxidation of DL-DOPA in PO activity of the serum of *P. jacquemontii*

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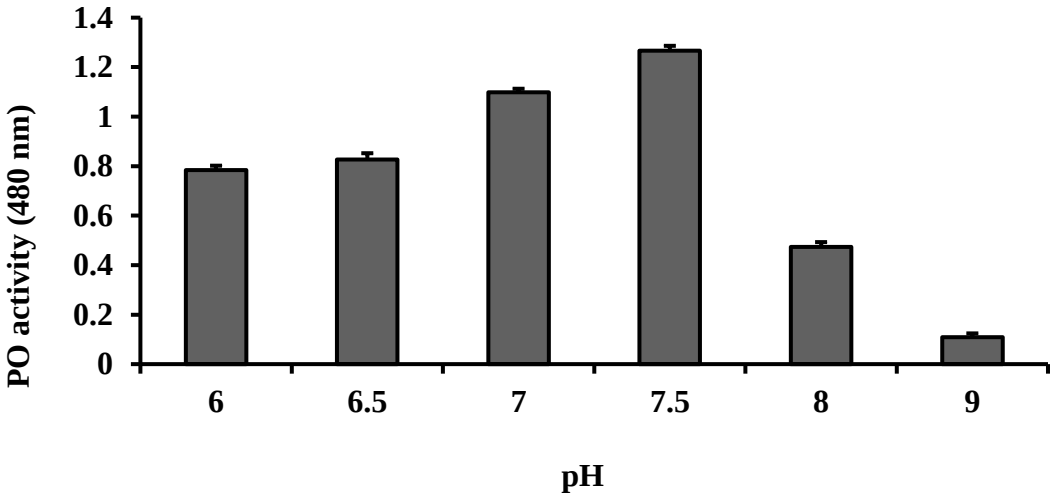

Fig 5: Effect of optimum pH on oxidation of DL-DOPA in PO activity of the serum of *P. jacquemontii*

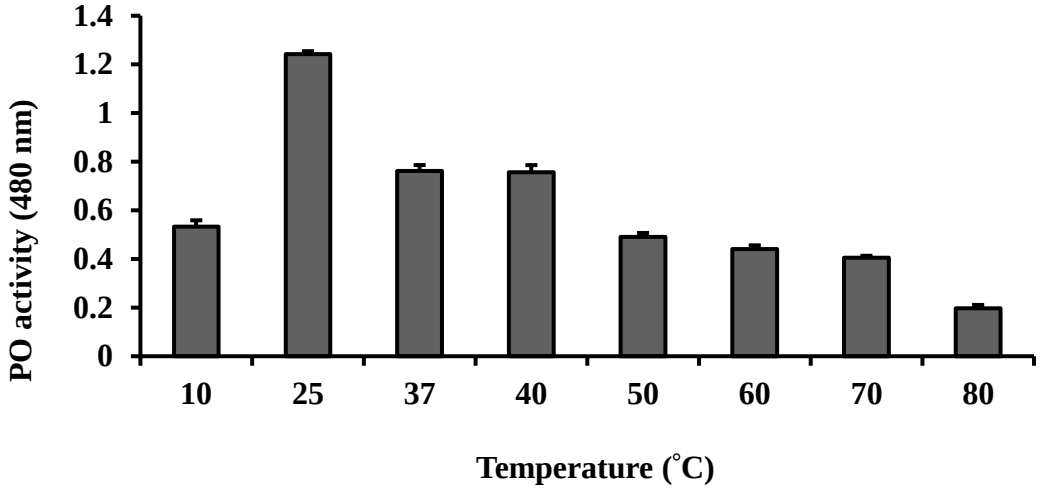

Fig 6: Effect of optimum temperature on oxidation of DL-DOPA in PO activity of the serum of *P. jacquemontii*

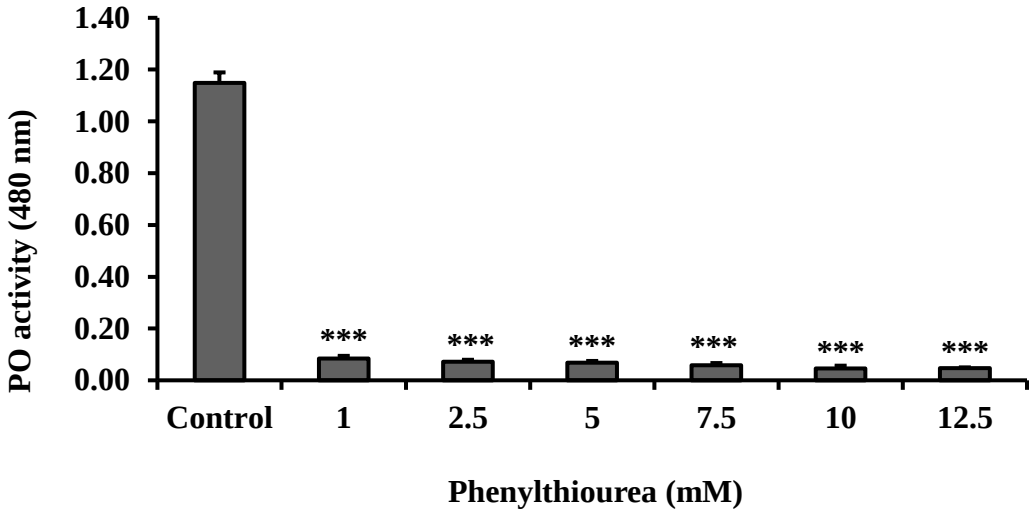

Fig 7: Inhibition of phenoloxidase activity using PTU in serum of the freshwater crab *P. jacquemontii*.

Asterisk indicates significant variation determined from the value obtained for a treatment to untreated control by one-way ANOVA significant at $^{***}P < 0.001$.

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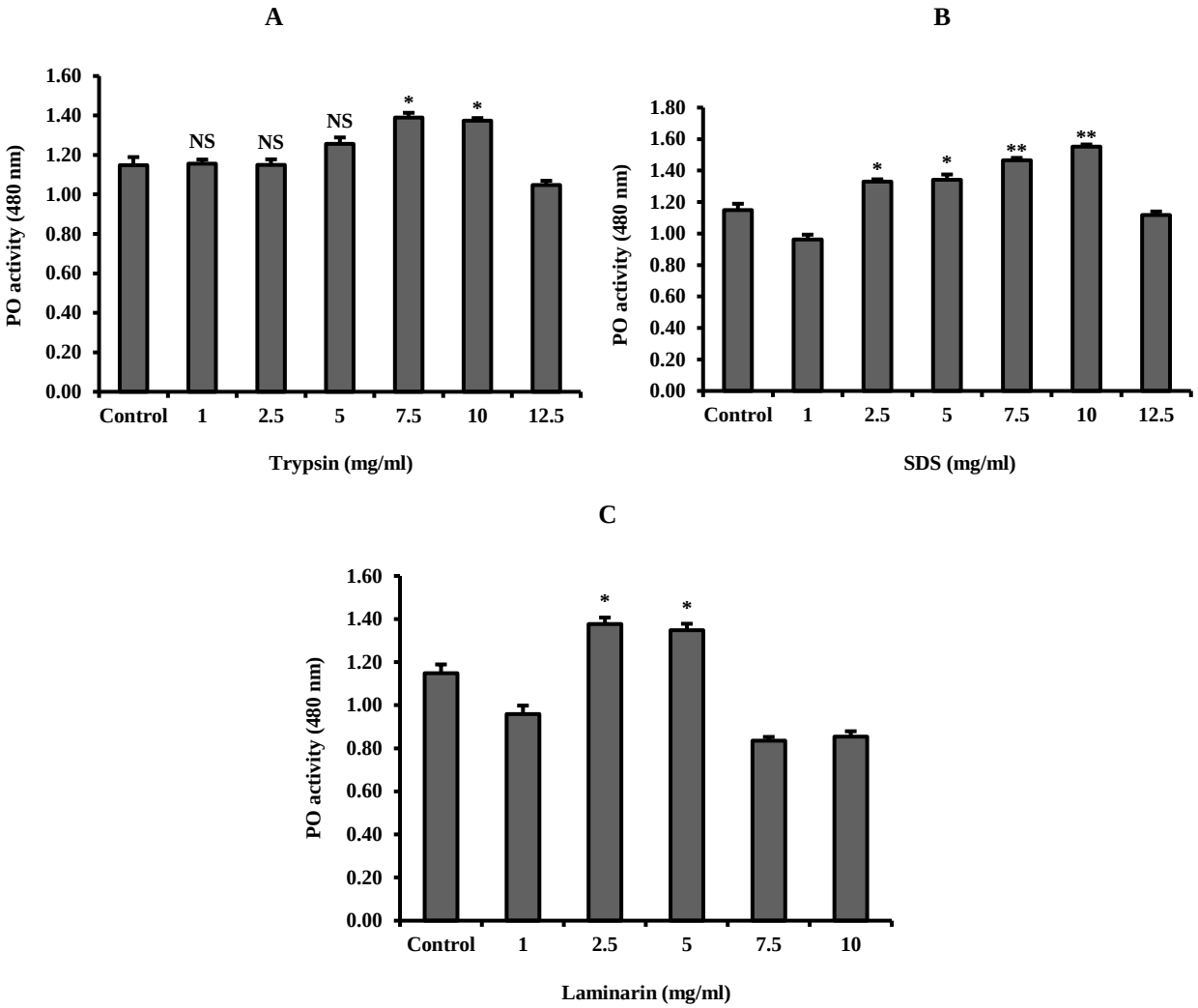

Fig 8: Activation of phenoloxidase activity using (A) trypsin, (B) SDS or (C) laminarin in serum of the freshwater crab *P. jacquemontii*

Asterisk indicates significant variation determined from the value obtained for a treatment to untreated control by one-way ANOVA significant at $^{*}P < 0.05$, $^{**}P < 0.01$. NS - not significant.