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DETECTION AND CHARACTERIZATION OF PHENOLOXIDASE FROM THE LARVAL HAEMOLYMPH OF BLOW FLY, *Hemipyrellia tagaliana* IN RESPONSE TO NON-SELF MOLECULES

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AUTHORS' CONTRIBUTIONS

All authors contributed to the study, conception and design. Material preparation, data collection and analysis were performed by authors TK, ITR and JS. The first draft of the manuscript was written by authors TK and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

In the deficiency of advanced immune mechanism in insects, the most common and one of the efficient methods used by insects in recognising pathogens or foreign non-self substances is the melanisation process. The primary enzyme involved in melanin biosynthesis by phenoloxidase (PO) thus plays a vital role in insect immunity. In the present study, larval haemolymph of the blowfly, (*Hemipyrellia tagaliana*) were used to demonstrate the presence of prophenoloxidase and its activating system. The haemolymph showed favourable results in the oxidation assays carried out to test the presence of prophenoloxidase using phenolic substrates. This PO activity was shown to be repressed by phenylthiourea, an inhibitor of PO activity, while the activity was increased when exposed to microbial components such as laminarin, LPS from various bacterial species and zymogen. The results indicated that this enzyme (PO) existed in zymogen state and was activated by proteolytic cleavage when exogenous proteases and detergents were introduced in the assay system. An increased PO activity in the presence of Ca^{2+} showed its calcium-dependent nature. These results conclude that a zymogen of phenoloxidase is found in the larval haemolymph of the *H. tagaliana*, which is activated by proteolytic cleavage when exposed to foreign non-self-substances and pathogens.

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1. INTRODUCTION

Insects represent one of the most successful groups of organisms in evolution accounting for nearly one million species and occupy almost all ecological niches. Consequently, they survive particularly with large diversity of bacterial pathogens. In insects, constitutive immunity is represented mostly by the background activity of the prophenoloxidase (proPO) cascade and by the immune capability of the circulating haemocytes [1]. This proPO system is a part of humoral immunity, comprising pattern recognition proteins, several serine proteases, their inhibitors, and end with the activation of the zymogen, ProPO [2,3]. Phenoloxidase (PO) is a copper containing enzyme [3] and heritable, found extremely in the cuticle, haemolymph and gut, protecting the animal from the invading pathogens.

In insects there are two types of PO *viz*. laccase-type PO and tyrosinase type-PO [4]. There is another type of proPO found to show high affinity towards diphenols known to be catechol oxidase-type [5]. One form is found in cuticle and the other in blood cells [6]. The laccase enzyme is responsible for sclerotization while the tyrosinase is involved in wound-healing. Upon injury, the proenzyme gets activated, giving rise to phenoloxidase that heals the wound. The phenoloxidase of the haemolymph is found to be a "sticky protein" and this could be the reason for its higher molecular weight due to the aggregation with other phenoloxidase enzymes and proteins [7]. This sticky nature is important because it leads to physical isolation of the foreign pathogen, forming melanisation layers [5].

Melanin produced as a defence mechanism in insects, are found to inhibit bacterial enzymes and fungal growth (fungistatic) [3]. Moreover, insects of pigmented phenotypes often possess strong immunity than pale phenotypes [8]. The key enzyme, PO involved in melanin biosynthesis also plays an important role in defence reactions of insects [3]. When triggered by foreign non-self-molecules, they are released by exocytosis converting the proPO to activated-PO. Melanisation involves hydroxylation of monophenol such as tyrosine to DOPA, oxidation of o-diphenols (DOPA) to o-quinones (dopaquinones) [9] and dehydrogenation of dihydroxyindole [10]. Insect PO has substrates belonging to two classes: monophenols - tyrosine, tyramine and catechol substrates - catechol (1,2- dihydroxybenzene), dopamine, DOPA, 4-methylcatechol, NADA (N-acetyldopamine), NBANE (N-acetylnorepinephrine), NBAD (N-ß-alanyldopamine), DOPAC (3,4-dihydroxyphenylacetic acid) [10].

In insects, dopamine is used as a precursor for melanin, instead of DOPA. For cuticular sclerotization, large amounts of catecholamines are stored in the blood of insects. These catecholamines are produced by acylating dopamine and decarboxylating DOPA. This reduces the free DOPA level and the soluble DOPA is outnumbered by soluble dopamine. Hence, the substrates dopamine and N-acyldopamines are preferred for POs [11]. Blowflies are the first insect to arrive on a dead body due to their attraction towards the odour of the fresh blood, particularly the blood from opened wound and body fluid of the carrion [12]. Calliphorids belonging to the genus *Hemipyrellia* are as well forensically important insects. The study organism in the present research work is the larvae of the blowfly *Hemipyrellia tagaliana* that live and grow in the compost by feeding on cattle dung. It is, therefore, very important that the larvae living in such hostile environment could have developed an efficient self-defence system against microbial infection. However, the role of self-defence in the larvae of this blowfly against microbes remains to be elucidated. Therefore, in the present work, detection and characterization of hemolymph phenoloxidase from the larvae of *Hemipyrellia tagaliana* was carried out for the first time.

2. MATERIALS AND METHODS

2.1 Experimental Animal

The larvae of blowfly, *Hemipyrelia tagaliana* were collected from Pattabiram, Tamil Nadu, India from the cow dung in which they feed and survive. The larvae were collected periodically in the dry season during 2019. The whole work was carried out at Unit of Entomology, Department of Zoology and University of Madras.

2.2 Haemolymph Collection

The collection of haemolymph was carried out by the protocol described by Tabunoki et al. [13]. The haemolymph collection procedure was slightly modified as described below:

The larvae were separated from cow dung using forceps and cleaned using distilled water and finally rinsed in 0.9% saline to remove the attached cow dung. The larvae were then stored in ventilated containers. The haemolymph was collected by

pricking the anterior region using sterile needle and was collected in a slide by applying pressure on the posterior end of the larvae. Approximately, 15 µl of haemolymph was collected from 10 larvae and was diluted using 100 µl of 50 mM Tris-HCl buffer (pH 7.5) in the presence of 115mM NaCl. Since the larvae were small, the haemolymph with 1:10 dilution was used for all the experiments.

2.3 Detection of pro PO Activation in Haemolymph of *H. tagaliana*

2.3.1 Determination of protein concentration

Concentration of protein in the haemolymph was determined by the method of Lowry et al. [14]. Absorbance was measured at 600 nm and a standard graph was plotted using BSA to detect the concentration of unknown samples.

2.3.2 Assay of PO activity

The phenoloxidase activity in haemolymph was assayed by mixing 100 µl of sample with 1 ml of 5 mM dopamine prepared in Tris-HCl buffer (pH 7.5) and incubated at 30°C for 5 minutes [15]. The dopachrome formed was measured using spectrophotometer at 480 nm against suitable reagent blank [16]. Among the substrates tested, dopamine showed the highest PO activity.

2.3.3 Oxidation of different dopamine concentrations by haemolymph

To know the optimal concentration of dopamine required for its maximum oxidation by haemolymph, 100 µl haemolymph was mixed with 1 ml dopamine solution of different concentrations (0.63 mM, 1.25 mM, 2.5 mM, 5.0 mM, 7.5 mM, 10.0 mM and 12.5 mM) and incubated for 5 minutes at 30°C [9]. The colour developed was measured spectrophotometrically at 480 nm against reagent blank.

2.3.4 Oxidation of dopamine by haemolymph at different pH

The ability of the haemolymph to oxidize dopamine at different pH was tested by incubating 100 µl haemolymph with 1 ml dopamine solution prepared in Tris-HCl buffer at different pH (6.0, 6.5, 7.0, 7.5 and 8.0) for 15 minutes at 30°C [17]. The colour developed was measured spectrophotometrically at 480 nm against reagent blank.

2.3.5 Oxidation of dopamine by haemolymph in different ionic strength of the buffer

The effect of buffer ionic strength on oxidation of dopamine by haemolymph was assessed by incubating 100 µl haemolymph with 1 ml dopamine prepared in Tris-HCl buffer (pH 7.5) with different ionic strength (0.05, 0.25, 0.5 and 1.0 M) at 30°C [9]. After 5 minutes, the optical density of each of these reaction mixtures was determined spectrophotometrically at 480 nm against reagent blank.

2.3.6 Oxidation of dopamine by haemolymph exposed to different temperatures

Haemolymph samples (each 100 µl) were held at temperatures ranging from 10°C to 80°C (10, 20, 30, 40, 50, 60 and 70, 80°C) for 15 minutes. Each sample was mixed with 1 ml of 5 mM dopamine and incubated for 5 minutes at 30°C [18]. The colour developed was measured spectrophotometrically at 480 nm against a reagent blank.

2.3.7 Incubation time for oxidation of dopamine by haemolymph

The optimal incubation time required for a rapid oxidation of dopamine by haemolymph was determined by incubating 100 µl haemolymph with 1 ml of 5 mM dopamine at 30°C [9]. The colour developed was monitored continuously up to 30 minutes in spectrophotometer at 480 nm against a reagent blank.

2.3.8 Effects of inhibitor on the oxidation of dopamine by haemolymph

In this experiment, 100 µl haemolymph was mixed with 50 µl of PTU of different concentrations (1 mM, 3 mM, 5 mM and 10 mM). In controls, these chemicals were substituted by Tris- HCl buffer (pH 7.5) and incubated for 15 minutes at 25°C [9]. The reaction mixture from the control and experiments were incubated with 1ml of 5mM dopamine for 5 minutes at 30°C. The optical density of both control and experiments were measured spectro-photometrically at 480 nm.

2.3.9 Exogenous proteases and detergents on haemolymph PO activity

Haemolymph (100 µl) was incubated with 50 µl of protease solution (trypsin, α-chymotrypsin and pronase-E; each 1mg.ml^{-1} and detergents (SDS; 6 mg.ml^{-1} and Triton X100) for 15 minutes at 30°C. In the controls, proteases and detergents were substituted by Tris-HCl buffer (pH 7.5). To each of these

incubation mixtures, 150 µl of 5 mM dopamine was added and further incubated for 5 minutes at 30°C [9]. The mixture was diluted with the Tris-HCl buffer in the ratio of 1:2 and the optical density of both control and experiments were measured spectrophotometrically at 480 nm.

2.3.10 Non-self molecules on haemolymph PO activity

The effect of non-self molecules on plasma PO activity was studied by incubating 100 µl haemolymph with 50 µl laminarin, bacterial LPS (*Escherichia coli*, *Salmonella abortus equi, Pseudomonas aeruginosa, Serratia marcescens, Klebsiella pneumoniae* and *Salmonella minnesota*) and zymosan solutions each at 10 µg concentrations for 15 minutes at 30°C. To each of these incubation mixtures, 150 µl of 5 mM dopamine was added and further incubated for 5 minutes at 30°C [19]. The mixture was diluted with the Tris-HCl buffer in the ratio of 1:2 and the optical density of both control and experiments were measured spectrophotometrically at 480 nm.

2.3.11 Calcium dependency in laminarin / pronase-E-induced haemolymph PO activity

Effect of different $CaCl_2$ concentrations on laminarin and pronase-E -induced PO activity was studied by incubating 100 µl haemolymph with 50 µl laminarin (1 $mg.ml^{-1}$) and 50 µl pronase-E separately prepared in Tris-HCl buffer (pH 7.5), containing one of the 10 different $CaCl_2$ concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mM) for 15 minutes at 30°C. To each of these incubation mixtures, 150 µl of 5 mM dopamine was added and further incubated for 5 minutes at 30°C [20]. The overall mixture was diluted with the Tris-HCl buffer in the ratio of 1:2 and the optical density of both control and experiments were measured spectrophotometrically at 480 nm.

2.4 Statistical Analysis

For the PO reactions, the results were analyzed using a one-way analysis of variance (ANOVA) in a SPSS Software Package (Ver. 21) for Windows. Values were considered significantly different at $p<0.05$.

3. RESULTS

3.1 Protein Estimation of Haemolymph from *H. tagaliana*

The concentration of protein estimated in the haemolymph of *H. tagaliana* was recorded as 14 mg/ml.

3.2 Oxidation of Different Phenolic Substrates by the Larval Haemolymph of *H. tagaliana*

The haemolymph from the larvae of *H. tagaliana* was found to oxidize different phenolic substrates at varying degrees. As shown in Fig. 1, the haemolymph exhibited high activity with a catechol substrate, dopamine (74.2 ± 0.8 $unit.mg^{-1}$). The relative activity of haemolymph with these phenolic substrates was in the decreasing order of dopamine > L-DOPA > tyramine. The reactivity of haemolymph was barely detectable with catechol. Since the highest degree of oxidation was obtained with dopamine, this substrate was used to detect PO activity in all subsequent experiments performed in this study.

3.3 Oxidation of Different Dopamine Concentrations by Haemolymph of *H. tagaliana*

As shown in Fig. 2, when the haemolymph was assayed with different concentrations of dopamine (0.63, 1.25, 2.5, 5.0, 7.5, 10.0 and 12.5 mM), the level of oxidation gradually increased with increasing substrate concentration and reached the highest activity at its concentration of 5 mM (77.7 ± 0.6 $unit.mg^{-1}$) and declined gradually at higher concentrations. Therefore, all the subsequent assays were performed with 5 mM dopamine.

3.4 Effect of pH on PO Activity in Haemolymph of *H. tagaliana*

The optimal conditions for haemolymph PO assay was further characterized by studying the influence of hydrogen ion concentration using Tris-HCl buffers of pH ranging from 6.0 to 8.0. The results presented in Fig. 3 showed that PO activity was maximum at pH 7.5 (102.4 ± 0.4 $unit.mg^{-1}$), which was significantly higher than those observed at other pH.

3.5 Effect of Ionic Strength of Tris-HCl Buffer on the PO Activity in the Haemolymph of *H. tagaliana*

When the oxidation of dopamine by haemolymph was tested with Tris-HCl buffers (pH 7.5) at different ionic strengths (0.01, 0.02, 0.05, 0.25, 0.5 and 1M), the haemolymph showed highest PO activity with 0.05 M (107.7 ± 0.7 $unit.mg^{-1}$) when compared to other ionic strengths tested as represented in the Fig. 4. Therefore, 0.05 M was used in the subsequent assays.

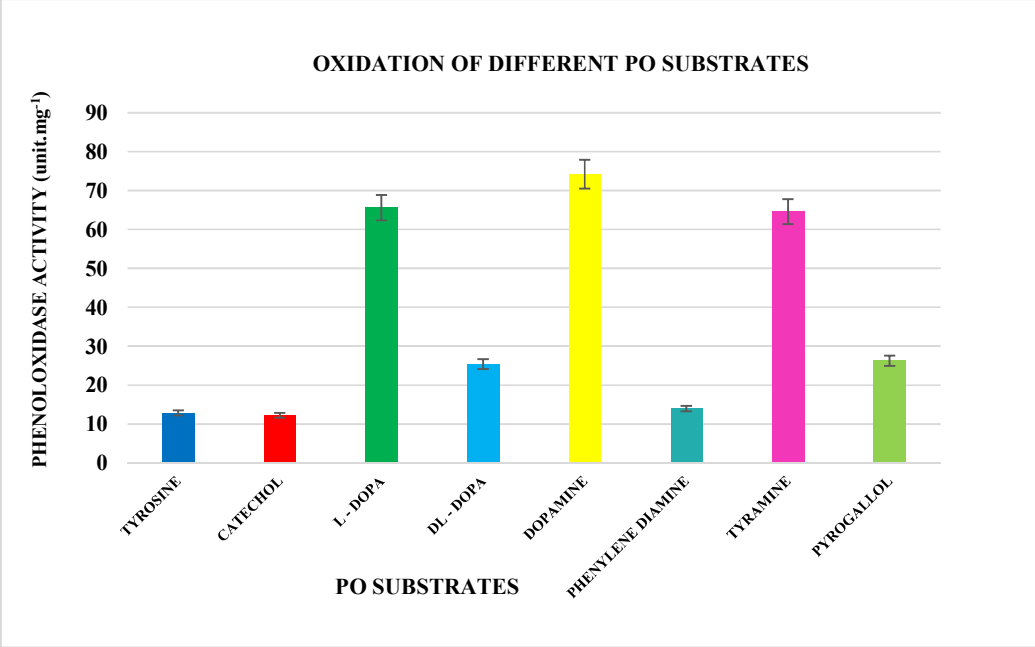

Fig. 1. Oxidation of phenolic substrates (each 5 mM) by the larval haemolymph of *Hemipyrellia tagaliana*
Values are shown as mean ± SD of 3 determinants using samples from different preparations

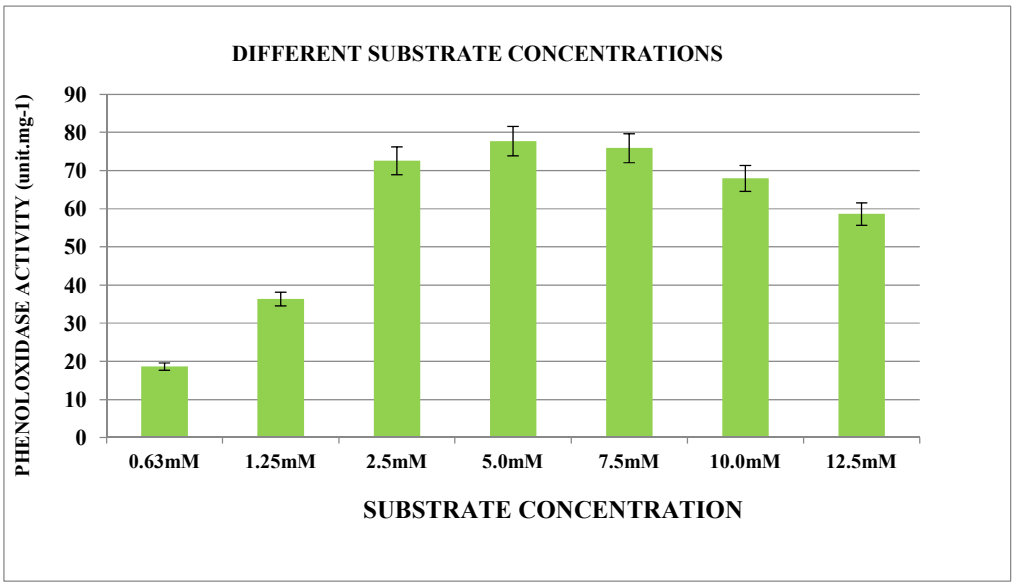

Fig. 2. Effect of various dopamine concentrations on its oxidation by the larval haemolymph of *Hemipyrellia tagaliana*
Values are shown as mean ± SD of 3 determinations using samples from different preparations

3.6 Effect of Temperature on the Oxidation of Dopamine by the Haemolymph of *H. tagaliana*

The enzyme activity of hemolymph pretreated at different temperatures was assayed using 5 mM dopamine under the conditions mentioned above in which the highest PO activity was observed at 0.05 M Tris-HCl buffer of pH 7.5 with 5 mM dopamine as substrate. The result presented in Fig. 5 revealed that the activity was increased from 10 to 20°C and a peak in the activity was observed at 30°C (122.7 ± 0.6

unit.mg^{-1}). Further increase in temperature decreased the activity. Therefore, all the PO assays in subsequent experiments were performed at a temperature of 30°C.

3.7 Effect of Incubation Time on the Oxidation of Dopamine by the Haemolymph of *H. tagaliana*

The data on the effect of incubation time on the haemolymph PO activity (Fig. 6) revealed a rapid oxidation of dopamine at 5 minutes of incubation of haemolymph with substrate. The PO activity was reduced at the higher incubation periods. Based on the result, 5 minutes incubation of haemolymph with substrate was considered as optimal incubation time for all subsequent PO assays.

3.8 Effect of Inhibitors on the Oxidation of Dopamine by the Haemolymph of *H. tagaliana*

The pre-treatment of haemolymph with phenylthiourea (PTU) gradually suppressed its ability to oxidize dopamine (Fig. 7). The inhibition of PO activity was higher at the concentration of 5 mM PTU.

3.9 Effect of Exogenous Activators and Detergents on the Oxidation of Dopamine by the Haemolymph of *H. tagaliana*

As shown in Fig. 8, preincubation of haemolymph with exogenous proteases such as trypsin (93.4 ± 0.8 unit.mg^{-1}) and pronase-E (137.7 ± 0.4 unit.mg^{-1}) enhanced the PO activity, while the activity was far less with α-chymotrypsin when compared to buffer-incubated control (67.8 ± 0.5 unit.mg^{-1}). The pre-treatment of haemolymph with SDS an anionic detergent, enhanced the PO activity (88.8 ± 0.7 unit.mg^{-1}). On the other hand, triton X-100, a non-ionic detergent significantly enhanced the enzymatic activity (150.1 ± 0.4 unit.mg^{-1}).

3.10 Effect of Non-self Molecules on the Oxidation of Dopamine by the Haemolymph of *H. tagaliana*

PO activity in the hemolymph was increased following incubation with non-self molecules namely *Salmonella minnesota, Klebsiella pneumoniae, Salmonella abortus equi, Serratia marcescens, Pseudomonas aeruginosa, Escherichia coli,* zymosan and laminarin. As represented in the Fig. 9, the activity was higher with *Serratia marcescens* (152.8 ± 0.8 unit.mg^{-1}), followed by zymosan (148.1 ± 0.6 unit.mg^{-1}) and laminarin (136.1 ± 0.7 unit.mg^{-1}).

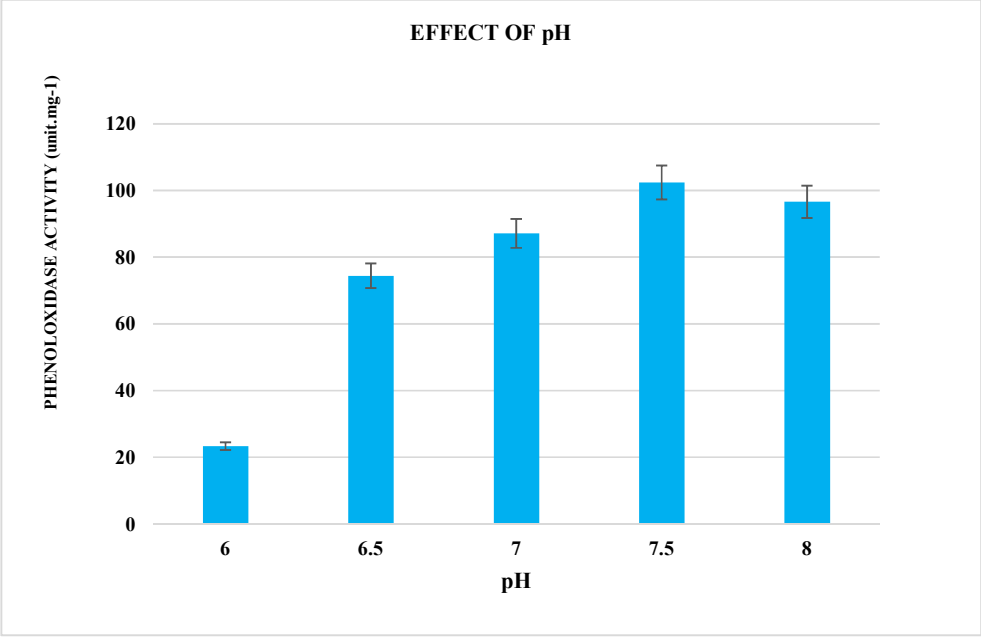

Fig. 3. Effect of pH of Tris-HCl buffer on oxidation of dopamine (5mM) by the larval haemolymph of *Hemipyrellia tagaliana*

Values are shown as mean ± SD of 3 determinations using samples from different preparations

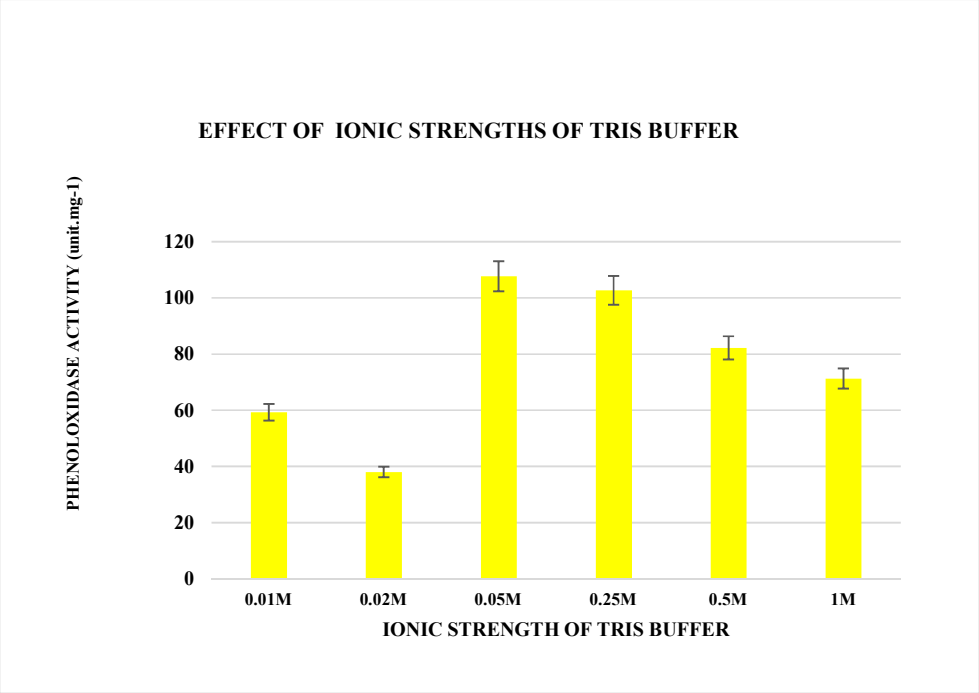

Fig. 4. Effect of ionic strength of Tris-HCl buffer on oxidation of dopamine (5 mM) by the larval haemolymph of *Hemipyrellia tagaliana*

Values are shown as mean ± SD of 3 determinants using samples of different preparations

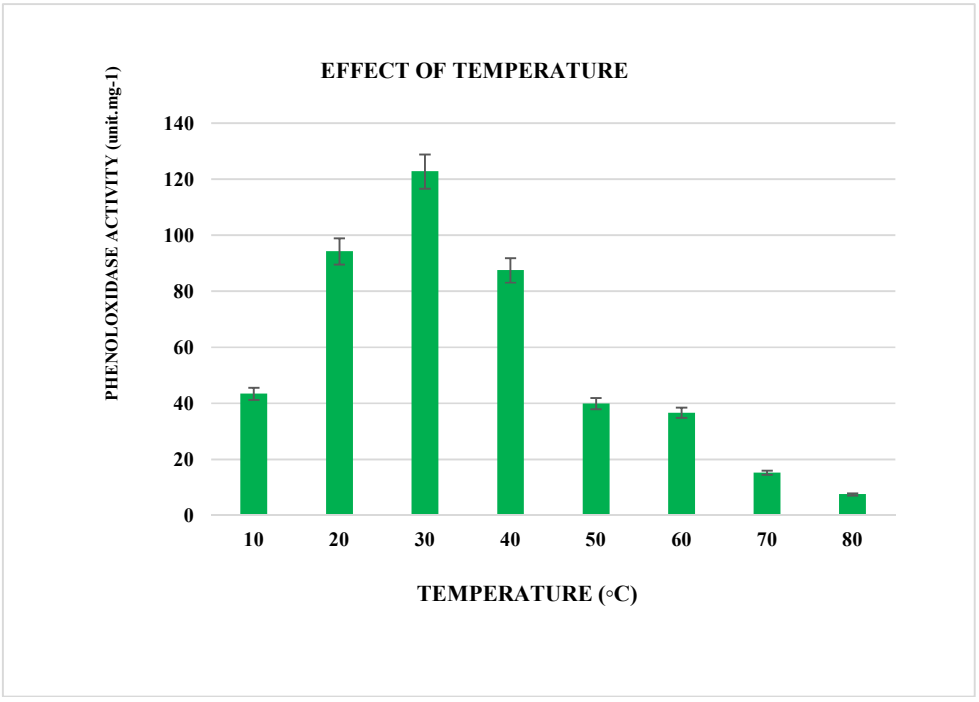

Fig. 5. Effect of temperature on oxidation of dopamine (5mM) by the larval haemolymph of *Hemipyrellia tagaliana*

Values are shown as mean ± SD of 3 determinants using samples of different preparations

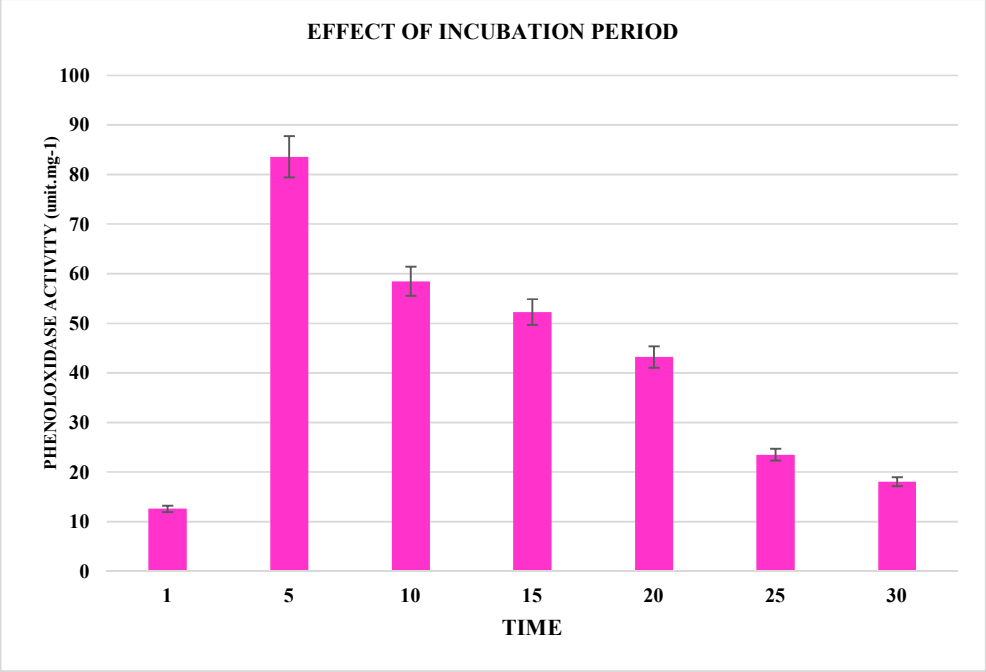

Fig. 6. Effect of incubation time on oxidation of dopamine (5mM) by the larval haemolymph of *Hemipyrellia tagaliana*

Values are shown as mean ± SD of 3 determinants using samples of different preparations

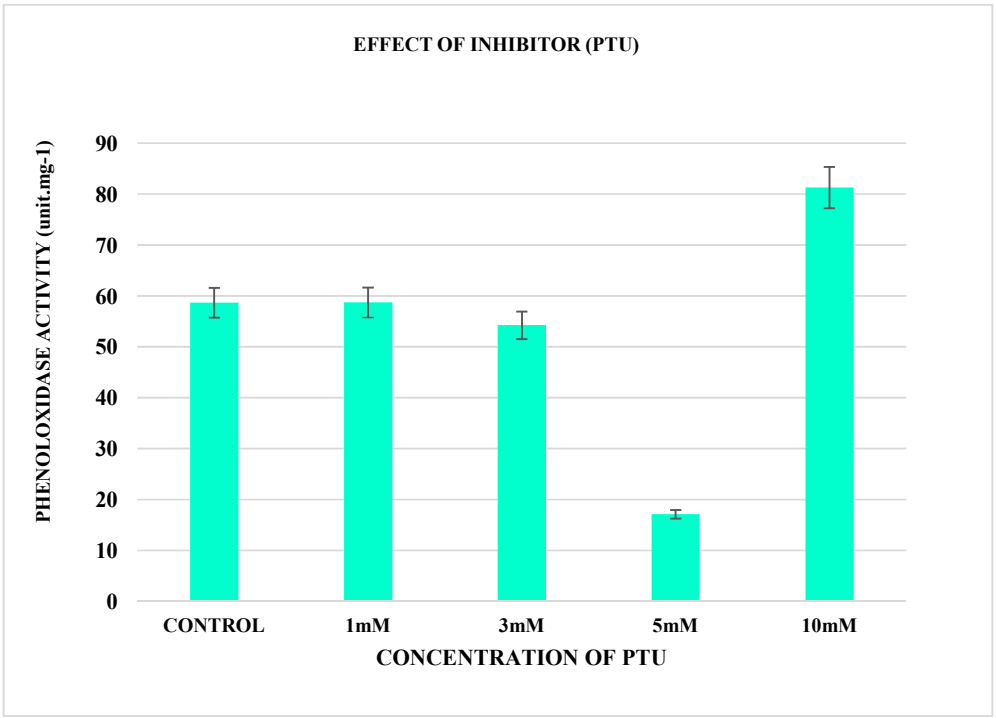

Fig. 7. Effect of inhibitors on oxidation of dopamine (5mM) by the larval haemolymph of *Hemipyrellia tagaliana*

Values are shown as mean ± SD of 3 determinants using samples of different preparations

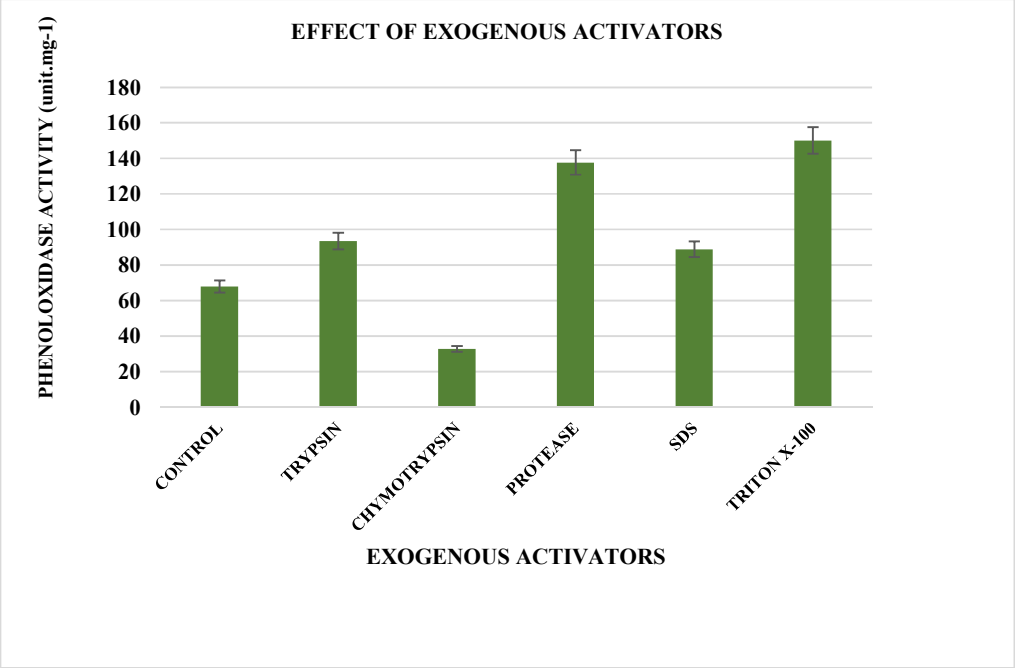

Fig. 8. Effect of exogenous activators on oxidation of dopamine (5 mM) by the larval haemolymph of *Hemipyrellia tagaliana*

Values are shown as mean ± SD of 3 determinants using samples of different preparations

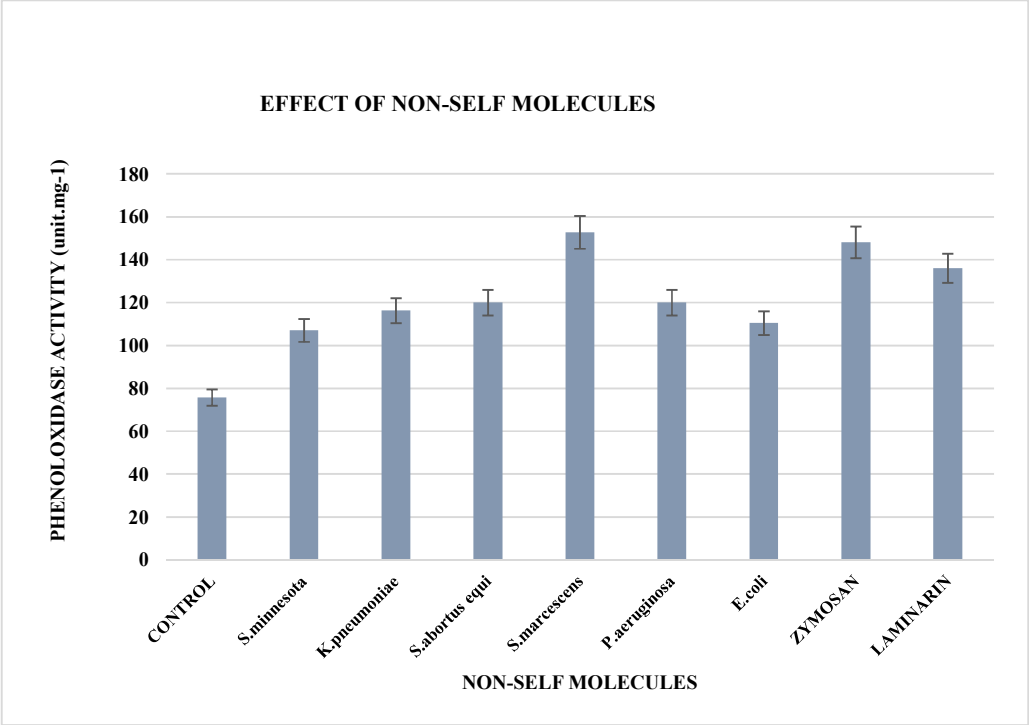

Fig. 9. Effect of non-self molecules on oxidation of dopamine (5mM) by the larval haemolymph of *Hemipyrellia tagaliana*

Values are shown as mean ± SD of 3 determinants using samples of different preparations

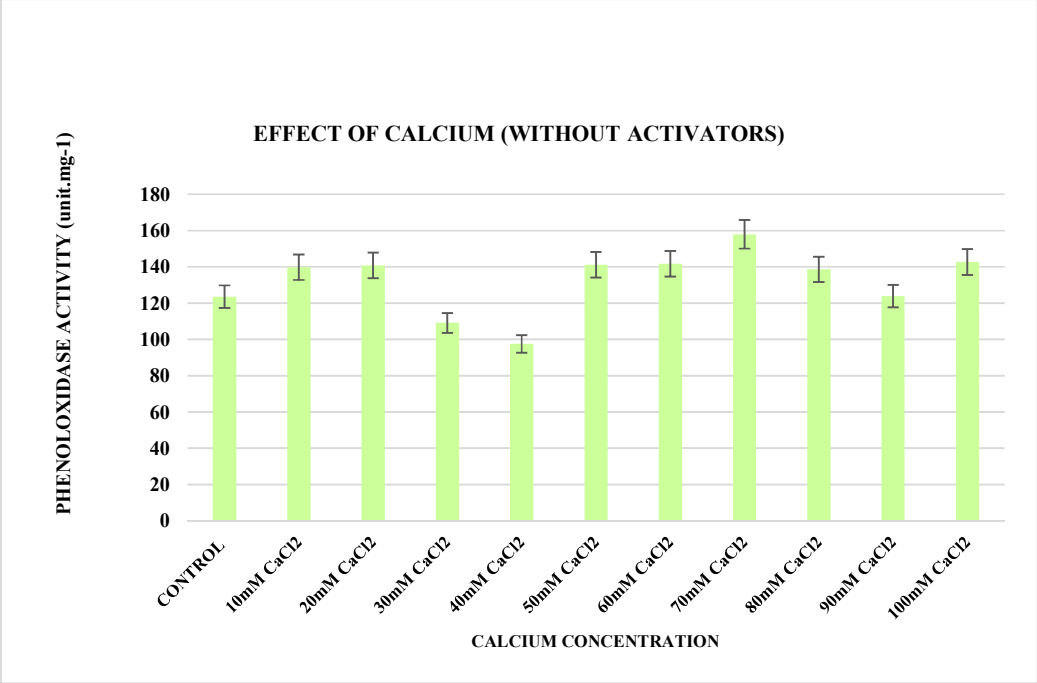

Fig. 10. Effect of calcium chloride on oxidation of dopamine (5 mM) by the larval haemolymph of *Hemipyrellia tagaliana*

Values are shown as mean ± SD of 3 determinants using samples of different preparations

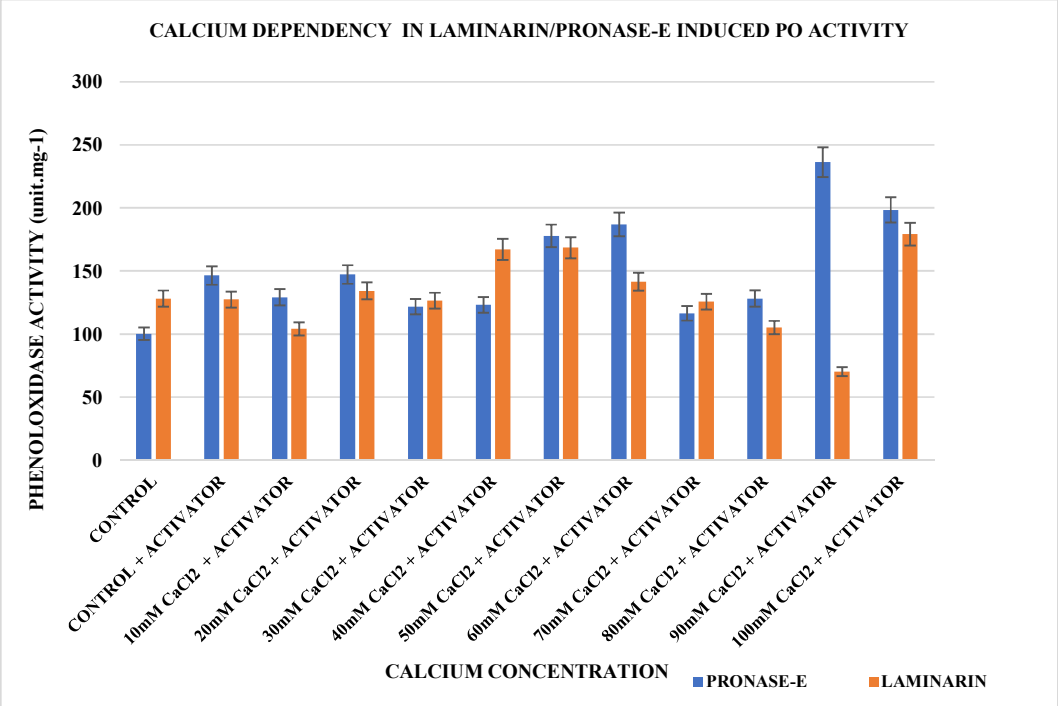

Fig. 11. Effect of calcium chloride on laminarin / pronase-E induced oxidation of dopamine (5 mM) by the larval haemolymph of *Hemipyrellia tagaliana*

Values are shown as mean ± SD of 3 determinants using samples of different preparations

3.11 Calcium Dependency on the Oxidation of Dopamine by the Haemolymph of *H. tagaliana*

The data on preincubation of haemolymph with different concentrations of $CaCl_2$ is presented in Fig. 10. Ca^{2+} enhanced the PO activity in the haemolymph at 10 and 20 mM $CaCl_2$ and reduced at the concentrations of 30 and 40 mM. The PO activity was observed high at 70 mM concentration of Ca^{2+}.

3.12 Calcium Dependency in Laminarin / Pronase-E Induced on the Oxidation of Dopamine by the Haemolymph of *H. tagaliana*

The data on preincubation of haemolymph with laminarin / pronase-E in the presence of different concentrations of $CaCl_2$ is presented in Fig. 11. Laminarin / pronase-E enhanced the PO activity in the haemolymph at 40-50mM $CaCl_2$ and at 90-100mM $CaCl_2$. The other $CaCl_2$ concentrations showed low activity.

4. DISCUSSION

The hemolymph of *H. tagaliana* showed high reactivity in oxidation of dopamine among several substrates tested, suggesting the preponderance of phenoloxidase enzyme and dopaminechrome isomerase activity. Dopamine is formed by the decarboxylation of DOPA by DOPA decarboxylase. The dopamine is further acted upon by phenoloxidase, followed by non-enzymatic reactions forming dopaminechrome and later DHI (dihydroxyindole). A separate dopaminechrome isomerase is needed for DHI formation, leading to melanin formation. Dopamine is a preferred substrate, since they need catecholamines (NAD and NBAD) for sclerotization [11]. The activity was low with phenylene diamine. This shows that the haemolymph possesses tyrosinase-type PO rather than laccase-type.

The PO activity in the haemolymph of *H. tagaliana* showed peak level towards dopamine at near neutral pH 7.5, which agrees well with the PO activity reported in the closely related dipteran *Musca domestica* (pH 7.4) [21] and also in other insects such as *Galleria mellonella* (Lepidoptera), pH 7.5 [22], *Spodeptera littoralis*; *Antheraea assamenesis* (Lepidoptera), (pH 7.5) [23,24] and *Melanoplus sanguinipes* (Orthoptera), pH 7.3 [25]. The optimal incubation time for PO activity found was 5 minutes, which indicates that the enzymatic PO reaction would able to oxidise the heamolymph at very short duration of time. This result is in agreement with those reported in *Aedes aegypti* [26]; *Holotrichia diomphalia* [15] and *Perna viridis* [9]. The optimal oxidation of dopamine was found to be at 5 mM substrate concentration and 50 mM tris-HCl at optimum temperature of 30°C. These results indicate that the haemolymph could enhance the enzymatic PO activity even at low concentration of substrates. Similar such results were recorded in Sydney rock oyster, *Saccostrea glomerata* [27]; *Octopus ocellatus* [17]; *Bactocera dorsalis* [18] and *Antheraea assamenesis* [24].

PTU, a known inhibitor of PO [28], reduced the oxidation of dopamine in the haemolymph of *H. tagaliana*. The inhibition of PO activity by PTU were reported in several invertebrate species like, *Lucilia cuprina* [29]; *Tenebrio molitor* [30]; *Spodoptera littoralis* [31]; *Saccostrea glomerata* [27]; *Crassostrea gigas* [32]; *Plodia interpunctella* [33]; *Apis melliferra* [16]and *Lymantria dispar* [34], thereby indicating that the oxidation of substrates was due to action of PO present in the haemolymph.

The enhancement of haemolymph PO activity upon pre-treatment with exogenous proteases such as trypsin and pronase-E clearly indicates that the tyrosinase-type PO could exists in proenzyme form and could be activated by proteolytic cleavage by these serine proteases [2] as reported previously in haemolymph of cockroach *Leucophaea maderae* [35]; *Spodoptera littoralis* [23]; *Dactylopius cocuus* [36], *Biomphalaria glabrata* and *B. alexandrina* [37]. Treatment of haemolymph of *H. tagaliana* with SDS enhanced the PO activity when compared to the control. This agrees with those reported on PO activity in in larvae of *Sarcophaga* and *Manduca* [38]; *Limulus Polyphemus* [39]; *Saccostrea glomerata* [27] and *Pandinus imperator* [40]. The initiation by SDS probably includes its binding and consequent induction of minor conformational change on the proenzyme leading to disclosure of its active site. From the above study, Triton X 100, an non-ionic detergent exhibited high PO activity when compared to that of SDS. This was similar to the observations seen in *Saccostrea glomerate*, in which haemolymph treated with Triton X-100 with dopamine as substrate showed increased activity [27].

Several earlier studies have shown that the microbial cell wall components such as laminarin, bacterial LPS and zymosan triggered *in vitro* activation of ProPO in the haemolymph, indicating the importance of PO in immune-recognition process [41,42,1]. In this present study, the activation of proPO of *H. tagaliana* was tested *in vitro* with laminarin, six different types of bacterial LPS and zymosan. The preincubation of haemolymph with laminarin a polymer of β-1,3

glucan and zymosan, significantly enhanced PO activity in hemolymph of *H. tagaliana* thereby indicating activation response of proPO to a non-self molecule. The observations in PO activity made in the previous reports such as *Locusta migratoria* [41]; marine mussel, *P. viridis* [9]; *Crassostrea gigas* [32] and *Helicoverpa armigera* [19] clearly correlated with the activation of haemolymph PO of *H. tagaliana* by the fungal cell wall components. Activation of PO in the haemolymph was carried using LPS from various bacterial strains. The result obtained indicated higher level of PO activity with LPS from *Serratia marcescens* out of the six bacterial LPS tested. The activation of PO with LPS from *E. coli* was also reported in *Locusta migratoria* [41], *Schistocerca gregaria* [43]; *Spodoptera littoralis* [23] and *Periplaneta americana* [44]. Our observations thus indicate that bacterial LPS effectively activated the proPO present in hemolymph of *H. tagaliana* and that this enzyme appears to show a selective response in activation by different LPS types tested. This selective response may involve receptor-mediated activation of proPO by LPS.

Calcium ions are identified as the necessary co-factor for laminarin-induced activation of proPO cascade in arthropods. The activation of proPO by non-self molecules requires Ca^{2+} in *Bombxy mori* [45]; *Eurygaster integriceps* [20] and crustaceans [46]. However, in *B.mori* the ProPO activating enzyme can convert proPO to PO, in the absence of calcium [45] which contrasts the crustacean proPO system, in which conversion in the absence of Ca^{2+} ions produces an inactive PO [46]. To check the role of activator induced hemolymph PO activity, the pronase-E substituted experiment was carried out. In insects and crustaceans, the proPO activation is prevented at high Ca^{2+} concentration, even when the activators are added. At low concentrations of Ca^{2+}, ProPO is activated after adding activators. Prolonged storage leads to spontaneous activation of ProPO [47]. On contrary, both laminarin and Pronase-E induced PO activity of the larval hemolymph of *H. tagaliana* with increase in $CaCl_2$ concentration at varied level, with a highest peak at a concentration of 100 mM $CaCl_2$.

The PO activity in the haemolymph was not affected even when exposed to the Ca^{2+} without the activation of non-self molecules as reported in *Spodoptera littoralis* [23] and *Schistocerca gregaria* [44]. Optimal activation of PO could be achieved without risk of spontaneous activation, which would occur at lower Ca^{2+} concentrations [47]. In the present study, the obtained results revealed that the Ca^{2+} helps in spontaneous activation of the enymatic PO without non self molecules. This shows the hemolymph of *H. tagaliana* posess significant enzymatic PO when treated with Ca^{2+} to perform the spontaneous activation of the PO system, which helps in the greater protection of the organism.

Finally, although phenoloxidase is known to be widely distributed throughout the animal kingdom, this study unambiguously demonstrates its presence as a proenzyme (proPO) in insect. The activation responses of this proenzyme in the hemolymph of *H. tagaliana* to exogenous proteases, microbial cell wall components, and its susceptibility to protease inhibitors in vitro resemble the proPO activation system of arthropods. The relationships in activation responses of the haemolymph proPO system, mainly to the components of microbial cell wall in animals belonging to different phyla, tend to imply a unifying biochemical mechanism for immune recognition among invertebrates. Furthermore, this study demonstrates a selective activation response of hemolymph proPO of *H. tagaliana* to different types of bacterial LPS. Functional studies on haemocytic proPO system would further resolve their relative roles in insect immune reactions.

5. CONCLUSION

Innate immune system in insects is composed of various specific and non-specific responses and triggered in the presence of pathogens. The enzyme phenoloxidase is one such activation mechanism to battle pathogenic infections. In the present study, proPO and its activation mechanism is explained in the larval haemolymph of *H. tagaliana*, for the first time, which live and grow on filthy organic compost by feeding on cattle dung. It is therefore, very important that the larvae living in such hostile environment could have developed an efficient self-defence system against microbial infection. Consequently, the activation responses of this proenzyme in the hemolymph of *H. tagaliana* to exogenous proteases, microbial cell wall components and its susceptibility to protease inhibitors were demonstrated through various *in vitro* experiments. The results concluded occurrence of innate immune response in this insect through phenoloxidase cascade and its importance in the self-defence against invading pathogens.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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