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Detection and substrate portrayal on the serum phenoloxidase activity from the grub of rhinoceros beetle, *Oryctes rhinoceros*

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Research Paper

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Abstract

Phenoloxidase (PO) is a significant biomolecule involved in humoral defence mechanism of invertebrates. Spontaneous melanization of insect haemolymph is the major hinderance for studying PO activity, as haemolymph was collected devoid of phenylthiourea. In the study, no visible melanization was observed in crude serum from the grub of *Oryctes rhinoceros* up to 30 min of incubation amongst crude haemolymph, diluted haemolymph, crude serum and diluted serum that were subjected to visual observation for spontaneous melanization reaction. Accordingly, crude serum was taken for evaluating PO activity. At the same time, as PO substrates tend to auto-oxidize and provide false optical density value, tris-buffered saline devoid of any substrates were used as blank for PO assays. The ideal wavelength at which maximum PO activity occurred for each substrate, namely, tyrosine, tyramine, dopamine, L-dopa, DL-dopa, catechol, protocatechuic acid and pyrogallol was determined as 407, 410, 429, 465, 403, 466, 428 and 400 nm, respectively. Additionally, time course of oxidation for each phenolic substrate by the serum PO were examined and DL-dopa was identified as the specific substrate for serum PO in the grub of *O. rhinoceros*. Furthermore, maximum PO activity was observed at 5 min of incubation for 10 mM of DL-dopa that was considered as optimum concentration. The ideal pH and temperature for serum PO activity was observed as 7.5 and 20°C, respectively. These results suggested that standardizing a suitable substrate is an essential prerequisite to evaluate the real PO activity of serum which might significantly fluctuate in each insect model.

Introduction

Insects were among the first successful air-breathing land animals that inevitably make earth adaptable to the founding and evolution of species of a higher order, including *Homo sapiens*, without which our planet would have devolved to an inert, lifeless, barren rock. They are the most diverse group of organisms making up to approximately 80% of all living beings and are ubiquitous in nature. This makes them susceptible to an innumerable number of pathogens and they overcome by having an extensive innate immune system that consists of physical barrier, humoral and cellular responses. One such extensive cascade is the phenoloxidase system. Enzyme phenoloxidase (PO) is a physiologically significant molecule in humoral defence mechanism of invertebrates (Cerenius *et al.*, 2008). It is a copper-containing enzyme, usually present in a zymogen form known as prophenoloxidase (proPO) (Sugumaran and Nellaiappan, 1990). They get activated by non-self and self-recognition mechanism of the host body (González-Santoyo and Córdoba-Aguilar, 2012). When highly conserved microbial structural motifs referred as pathogen-associated molecular patterns (PAMPs) get recognized by pattern recognition receptors (PRRs) that are present in the host body, it triggers the activation of a cascade of serine proteases which further activates PO activating enzymes and, therefore, the conversion of the proenzyme takes place from proPO into PO (Cerenius *et al.*, 2008; Baruah *et al.*, 2019). Activated PO catalyses two kinds of reactions, namely, hydroxylation of mono-phenols and its oxidation to o-diphenols and further oxidation of o-diphenols to o-quinones, then the quinones are non-enzymatically polymerized to melanin (Hung and Boucias, 1996). During this reaction, various toxic melanin derivatives like dopaquinone, indole-quinone and semi-quinone are released. All these have cytotoxic properties damaging invading pathogens (Vavricka *et al.*, 2010). Activated PO then involves in the processes like melanization (Ashida and Brey, 1998), sclerotization (Sugumaran and Nellaiappan, 2000; Liu *et al.*, 2010), encapsulation (Sugumaran, 2002; Strand, 2008), nodulation (Mullen and Goldsworthy, 2003) and wound healing (Ashida and Brey, 1995; Sugumaran *et al.*, 1996).

Adults of *Oryctes rhinoceros* (Linnaeus, 1758) commonly known as Asiatic rhinoceros beetle or coconut rhinoceros beetle, is one of the most notorious pests of coconut palm (*Cocos nucifera*) and African oil palm (*Elaeis guineensis*) (Huger, 1966; 2005) belongs to the superfamily Scarabaeoidea of order Coleoptera. It is also a minor pest of a number of other crop plants such as *Acanthophoenix rubra* (barbel palm), *Ananas comosus* (pineapple), *Arenya pinnata* (sugar palm), *Musa species* (banana), *Saccharum species* (sugarcane), *Phoenix sylvestris* (wild date palm), *Causuarina* (Austrian pine), etc. (Gressitt, 1953). Thus, there are 29 host plant genera for *O. rhinoceros*, of which, 16 are palm varieties (Goonewardena, 1958). Although the adults are serious pest of palm crops, grubs from *O. rhinoceros* are increasingly known for bioconversion of wastes. Grubs are voracious feeders and they convert a higher proportion of compost into biomass (Ganesh *et al*., 2021). The grubs usually are found in dead standing coconut palms, fallen coconut logs and rotting coconut stumps and also in piles of sawdust and cow dung manure (Gressitt, 1953). As a result, it is believed that these grubs that are living in such hostile environments must have developed an efficient self-defence system against microbial infections (Shelomi *et al*., 2019; Bhuvaragavan *et al*., 2023).

Therefore, the grub of the insect *O. rhinoceros*, is chosen as a potential model for evaluating PO activity. Although, crude PO activity was reported in *O. rhinoceros*, it was limited only to the cuticular level (Jeyaraj *et al*., 1986; Longankumar *et al*., 1996). The circulatory system of insects is regarded as the significant site of immune responses toward pathogenic invasion. Information pertaining to circulatory PO in the grub of *O. rhinoceros* is absent and needs a serious assessment. Also, a sufficient amount of haemolymph can be easily collected from the grub of *O. rhinoceros*, the time taken for auto-oxidation and melanization of collected haemolymph is long enough to carry out the PO assay in optimized sample setup arrangements. Thus, the objective of the study is to identify the desired substrate through a suitable protocol to evaluate circulatory phenoloxidase activity of *O. rhinoceros* at the larval stage. Above all, deciphering the PO features in this model insect could facilitate framing gene knock-out protocol for phenoloxidase, thus eventually providing a heads-up in the control or management of *O. rhinoceros.*

Materials and methods

Experimental animal, haemolymph collection and serum preparation

Third instar grubs of *O. rhinoceros* (about 5 cm in length) were collected from the local farms of Tiruvallur district, Tamil Nadu, India, from the cow dung pits and dead standing coconut palms and reared in plastic troughs in the laboratory by providing cow dung as a source of feed. The grubs were cleaned using distilled water and rinsed in 0.9% saline to remove the strung-out cow dung. Haemolymph was collected devoid of phenylthiourea (PTU) (used to inhibit melanization) by gently cutting one of the pro-legs using sterile scissors in 1.5 ml micro-centrifuge tube held on ice. Then, the collected haemolymph was vortexed for 1 min and centrifuged at 12,000 rpm for 5 min. The serum was collected and used for the assays. The concentration of protein present in the serum was determined by following the method of Lowry (1951) and absorbance was measured at 600 nm. A standard graph was plotted using BSA to detect the concentration of unknown samples.

Preparation of substrate solutions

PO substrates such as tyrosine (L-3 [4-hydroxyphenyl] alanine) (99%), tyramine (99%), L-dopa (DL-3,4-dihydroxyphenylalanine) (99.0%), DL-dopa (DL-3,4-dihydroxyphenylalanine) (99%), dopamine (98%), catechol (99%), pyrogallol (99%) were purchased from HiMedia, Mumbai, India and protocatechuic acid (97%) was purchased from Sigma-Aldrich, Munich, Germany. The 10 mM concentration of each substrate were prepared by dissolving required quantities of respective substrates in 5 ml of tris-buffered saline (TBS) (50 mM tris, 90 mM NaCl, pH 7.5) (Radha *et al*., 2013).

Examining the time duration of melanization and selection of appropriate test sample for PO spectroscopic studies

To check spontaneous melanization and velocity of melanization upon different time intervals and to select appropriate reaction sample for studying PO assay, crude haemolymph, diluted haemolymph (1:5 dilution in TBS), crude serum, diluted serum A (1:1 dilution in TBS) and diluted serum B (1:1 dilution in TBS containing $CaCl_2$) were subjected to visual observation by maintaining the above samples at 24°C and periodically recording the intensity of melanization reaction.

Standardization of appropriate blank for PO spectroscopic studies

Initially, TBS buffer devoid of any substrates was taken as a blank for characterizing the suitable substrate of serum PO. Note: Buffer containing substrate solution cannot be used as the blank, unless a suitable substrate of serum PO is standardized. This is because PO substrates tend to auto-oxidize upon time and provide false positive optical density values every time when the readings are taken.

Standardization of desirable phenolic substrate of serum PO

Evaluation of absorption maxima of each substrate

The optimum wavelength at which maximum PO activity eventuated for each substrate was ascertained by incubating crude serum (100 μl) with an equal volume of each substrate solution (100 μl) for 15 min. Then, the reaction mixture was diluted to 300 μl using TBS and the absorption maxima (λ max) of the reaction mixture of each substrate were scanned between the wavelength ranging 380 and 800 nm.

Concurrently, auto-oxidation of each substrate during the reaction period was also checked by incubating each substrate with the TBS buffer (1:1 ratio) for the same 15 min and further diluted to 300 μl using TBS buffer and finally OD of the standing substrate was measured at the respective λ max. This was carried out for all the eight substrates, *viz*., monophenols (tyrosine, tyramine), diphenols (dopamine, L-dopa, DL-dopa, catechol and protocatechuic acid) and polyphenols (pyrogallol), each at 10 mM concentration.

Evaluating time course of oxidation of each PO substrate by serum PO

Serum was freshly prepared from the haemolymph of the third instar grub of *O. rhinoceros* and without any delay it was diluted to 1:1 ratio with freshly prepared substrate solution (each 10 mM concentration). The reaction mixture was gently mixed and incubated for various time intervals of 5, 10, 15, 30, 45 and 60 min. After respective time of incubation, the reaction mixture was evenly mixed and diluted to 300 μl using TBS buffer and the

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optical density was measured at the substrate specific λ max. This provided the absorbance value for oxidation of substrate by serum PO.

As mentioned previously, auto-oxidation of the substrate during the same time periods was examined by diluting the substrate solution with TBS buffer in 1:1 ratio (200 μl). The reaction mixture was then incubated for various time intervals of 5, 10, 15, 30, 45 and 60 min. After each time of incubation, the reaction mixture was further gently mixed and diluted to 300 μl using TBS buffer and the optical density was measured at the respective λ max. This provided the absorbance value for auto-oxidation of substrate during the incubation time.

Finally, the oxidation of substrate by serum PO alone can be calculated using the formula,

$$\text{Oxidation of substrate by serum PO} = (\text{OD value of substrate} + \text{serum}) - (\text{OD value of substrate} + \text{TBS buffer})$$

Then, the velocity of the reaction at every incubated time can be calculated using the following formula:

$$\text{REACTION VELOCITY} = \frac{\text{OD at absorption maxima}}{\text{Incubation time at minutes}}$$

This was conducted for all the eight substrates tested, namely, monophenols (tyrosine, tyramine), diphenols (dopamine, L-dopa, DL-dopa, catechol and protocatechuic acid) and polyphenols (pyrogallol).

Oxidation of different DL-dopa concentrations by serum

To know the optimal concentration of DL-dopa required for its maximum oxidation by serum, 100 μl of serum was mixed with 100 μl of DL-dopa substrate solution (1:1 concentration) at different concentrations (5.0, 7.5, 10, 12.5 and 15 mM) and incubated for 5 min at 25°C. The auto-oxidation of the substrate during the same time period was examined by diluting the substrate solution with TBS buffer in an 1:1 ratio. Finally, both the test samples were diluted to 300 μl using tris-HCl buffer and the colour developed was measured spectrophotometrically at 403 nm against a reagent blank, tris-buffered saline (50 mM, pH 7.5). Then, the oxidation of the substrate by the serum and the velocity of the reaction mixture were calculated using the above-mentioned formula.

Oxidation of DL-dopa by serum at different pH

The ability of the serum to oxidize DL-dopa at different potential of hydrogen ion concentration (pH) was examined by incubating 100 μl serum with 100 μl (10 mM) of DL-dopa substrate solutions (1:1 ratio) taken from 5 ml stock prepared at different pH (6.0, 6.5, 7.0, 7.5, 8.0 and 9.0) for 5 min at 25°C. The quantum of coloured product formed upon incubation was measured spectrophotometrically at 403 nm by diluting the sample to 300 μl against reagent blank TBS that was made at its respective pH for each assay. Further, the reaction velocity of each reaction mixture at different pH was calculated using the above-mentioned formula.

Oxidation of DL-dopa by serum at different temperatures

Serum samples (each 500 μl) were held at temperatures ranging from 10 to 60°C (10, 20, 30, 40, 50 and 60°C) for 30 min. After incubation, each sample was centrifuged at 12,000 rpm for 1 min and the serum was collected. 100 μl of serum was pipetted out and mixed with 100 μl of 10 mM DL-dopa substrate solution prepared at pH 7.5 in 1:1 ratio and incubated for 5 min at 25°C. Then, the reaction mixture was diluted to 300 μl and the colour developed was measured spectrophotometrically at 403 nm against a reagent blank.

Results

Estimation of protein concentration:

The protein concentration of serum devoid of PTU in the grub of *O. rhinoceros* was estimated to be 10.12 mg ml^{-1}.

Examination of the time duration of melanization and selection of appropriate test sample for PO spectroscopic studies:

Melanization reaction showed shades of brown colour when stored at 25°C (table 1). The occurrence of melanization was indicated by increase in colour intensity in all the four test samples upon visual observation. The OD value measured spectrophotometrically at 492 nm for each test sample found to increase upon time, further confirming the above results (Table S1). The intensity of colour formed due to melanization was much higher in haemolymph (fig. 1), diluted haemolymph (fig. 2) compared to other three samples. In the case of diluted serums A and B (fig. 3),

Table 1. Intensity of melanization reaction in various haemolymph preparations of grub of *O. rhinoceros*.

Time period of incubation (25°C)	Haemolymph	Diluted haemolymph	Crude serum	Diluted serum A	Diluted serum B
Soon after collection	-	-	-	-	-
15 min	+	+	-	-	-
30 min	++	++	-	-	-
1.0 h	+++	+++	+	+	+
1.5 h	++++	++++	+	+	+
2.0 h	++++++	+++++	++	++	++
3.0 h	++++++	+++++	+++	++	++
4.0 h	+++++++	++++++	++++	+++	+++

The number of '+' sign in the table indicates increase in spontaneous melanization reaction (formation of black colour) in the different preparation of hemolymph for the observed period of 4 h. '-' means no melanization (colourless).

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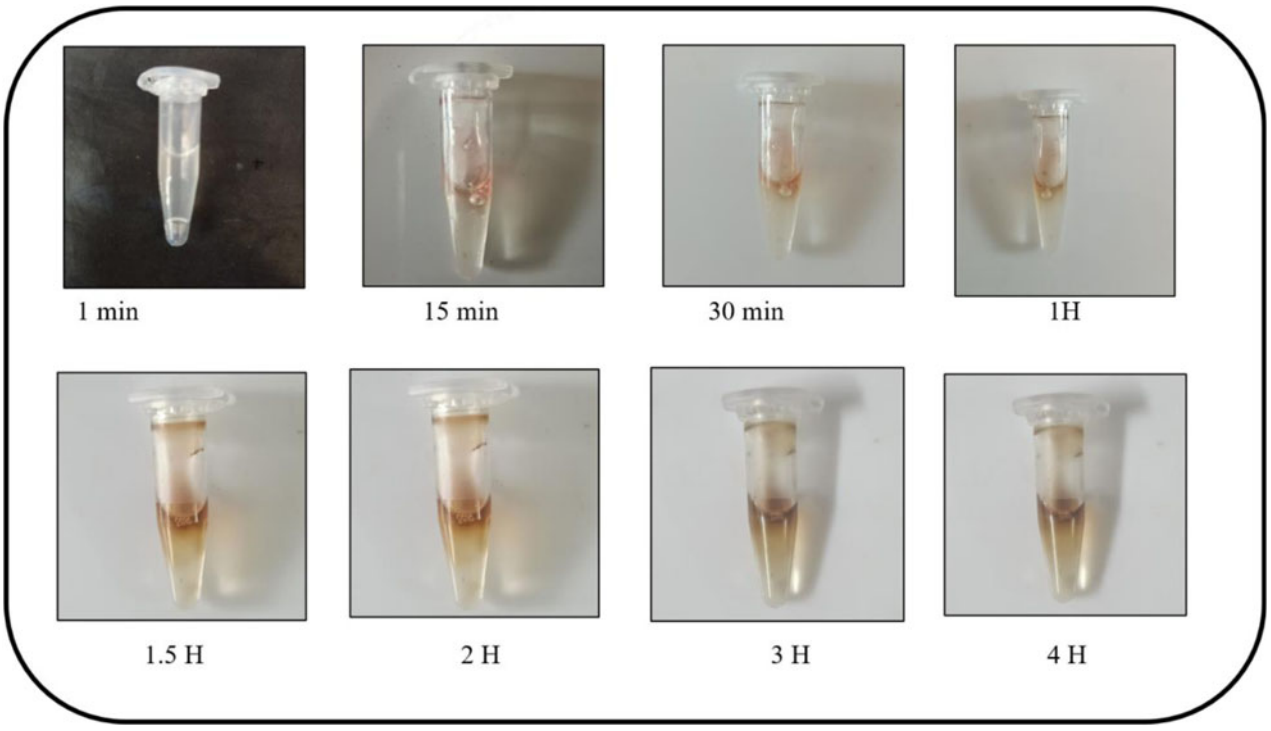

Figure 1. Spontaneous melanization reaction of grub haemolymph of *O. rhinoceros* on various time intervals.

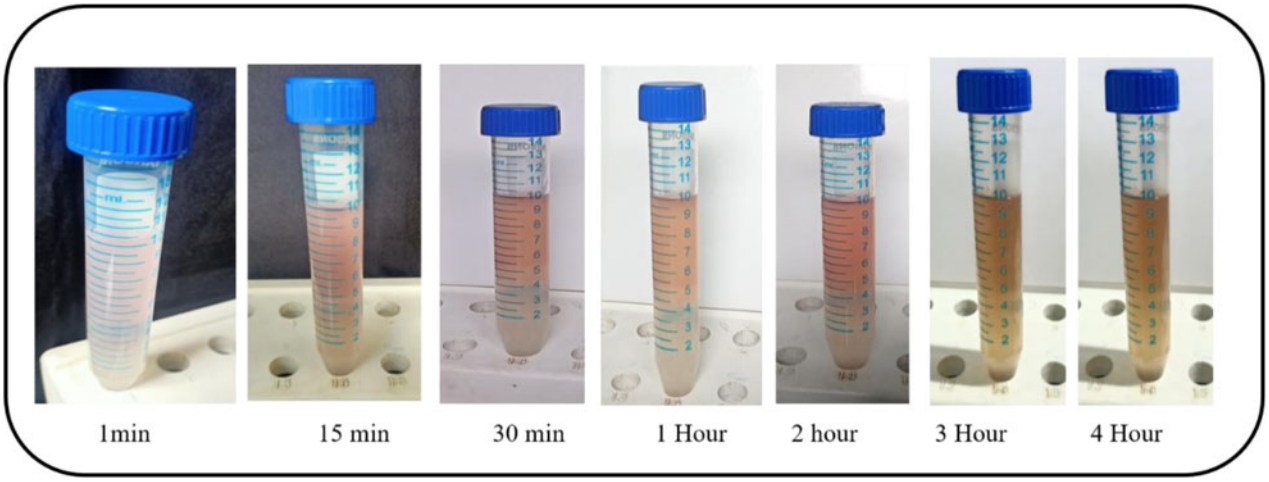

Figure 2. Spontaneous melanization reaction of 1:5 diluted grub haemolymph of *O. rhinoceros* on various time intervals.

the visually observed colour intensity was same. All these observations indicated that preferably crude serum could be used for evaluating PO activity.

Standardization of desirable phenolic substrate for serum PO activity

Evaluation of absorption maxima (λ max) for each substrate

The serum separated from the haemolymph of *O. rhinoceros* was found to oxidize different substrates at varying degrees. The λmax for coloured product formed upon oxidation of each phenolic substrate by serum (fig. 4) was examined. The optimal wavelength for the substrates, which include monophenols (tyrosine and tyramine), diphenols (dopamine, L-dopa, DL-dopa, catechol, and protocatechuic acid), and polyphenols (pyrogallol), was, therefore, determined as 407, 410, 429, 465, 403, 466, 428 and 400 nm, respectively (Radha *et al.*, 2013). Further, the auto-oxidation of the substrates during the same period of incubation was also measured and reported for all the eight substrates at their respective λ max.

Evaluation of time course of oxidation of each substrate by serum PO and selection of desirable phenolic substrate of serum PO

In all the substrates tested, the reaction velocity was maximum when the reaction mixture was subjected to 5 min of incubation,

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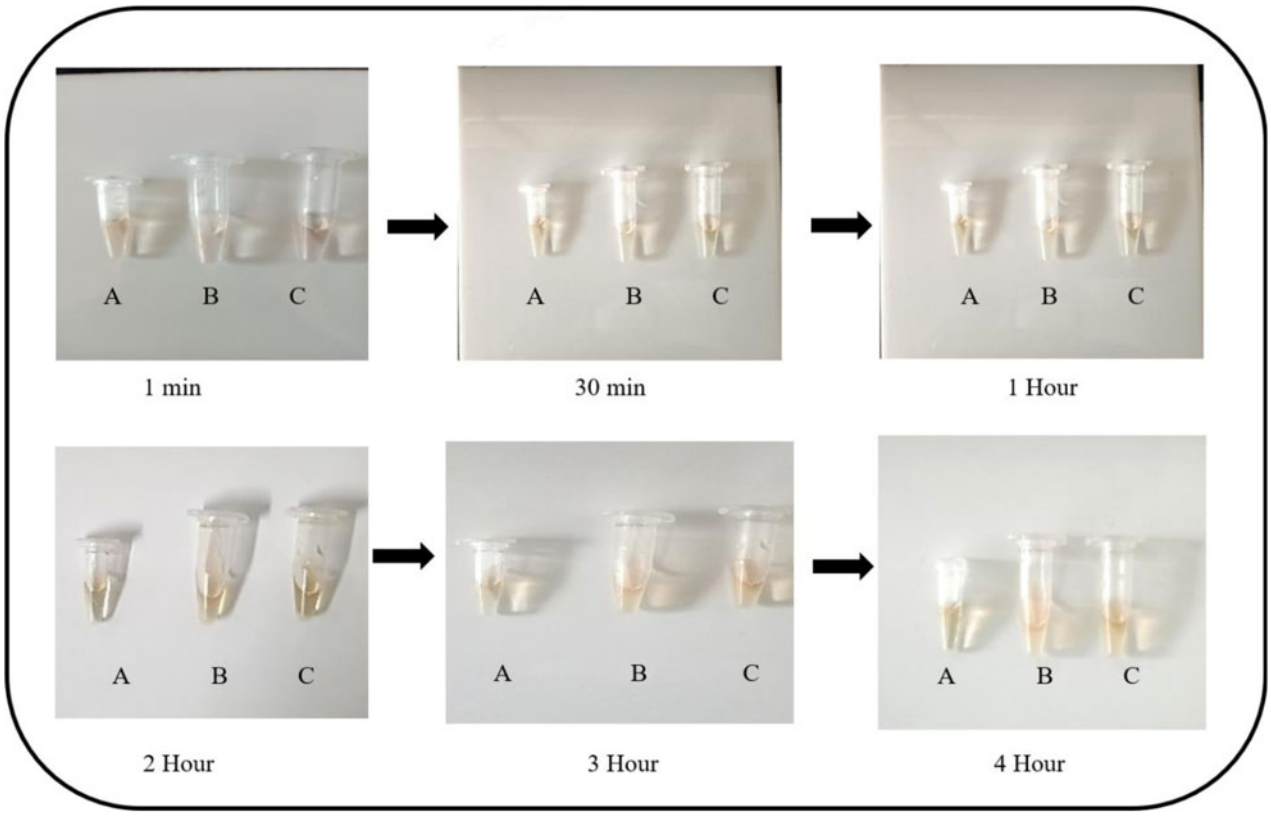

Figure 3. Spontaneous melanization reaction of serum, diluted serum A and B on different time intervals from the grub of *O. rhinoceros*. A-Crude serum, B-Diluted serum A (1:1 dilution with tris-buffered saline), C-Diluted serum B (1:1 dilution with tris-buffered saline containing calcium chloride).

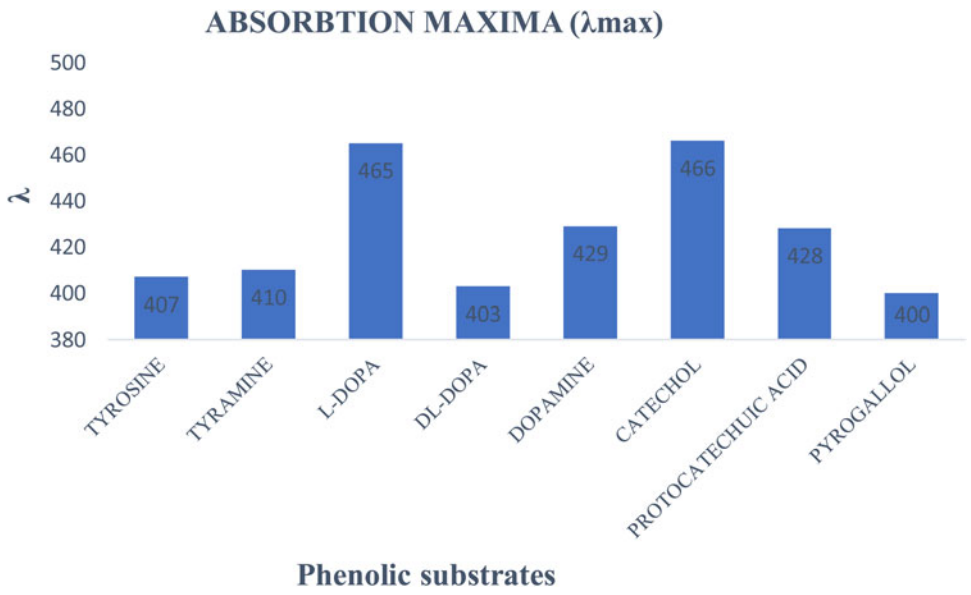

Figure 4. Absorption maxima for each phenolic substrate tested on serum PO activity in the grub of *O. rhinoceros* between λ 380 and 800 nm. Values represent mean ± SD of three determinations.

following which the velocity of serum PO activity decreased with sharp drop after 10 min of incubation. This showed that the influence of incubation time on reaction velocity to a great extent and thus, an incubation time of 5 min was considered as optimum to obtain maximum reactivity by the PO present in the serum. Based on these findings, the relative activity of serum PO with the phenolic substrates at 5 min of incubation were in the order of pyrogallol > DL-dopa > tyramine > L-dopa > dopamine > tyrosine > protocatechuic acid > catechol (fig. 5). Among the substrates tested, the serum PO exhibited a high level of oxidation with a

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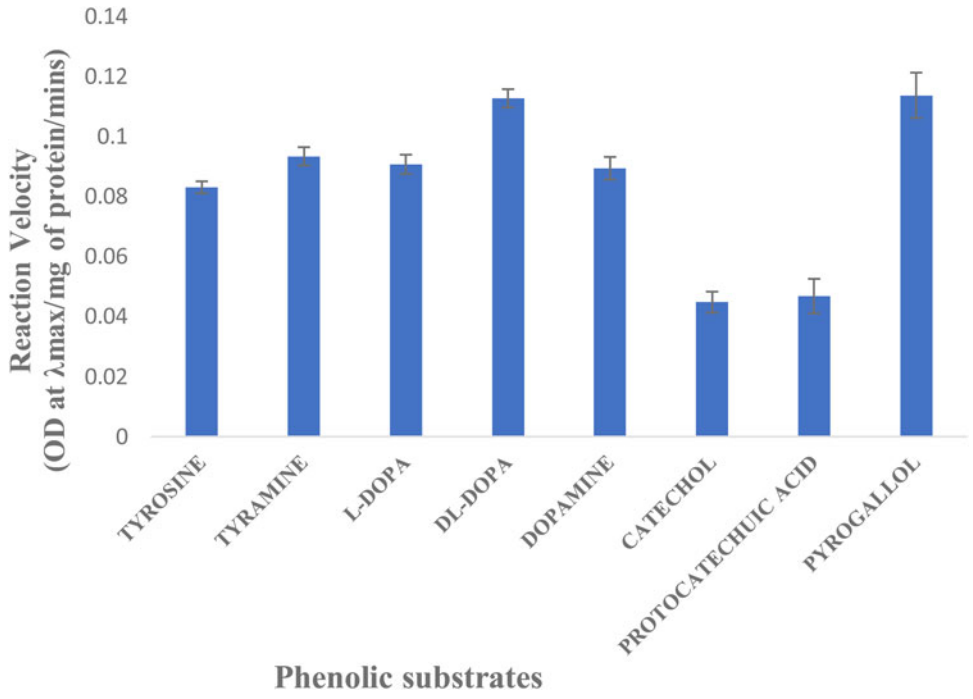

Figure 5. Effect of different phenolic substrates on the reaction velocity of serum PO activity in the grub of *O. rhinoceros*. Values represent mean ± SD of three determinations.

polyphenolic substrate, pyrogallol and the low level of activity with catechol, a diphenolic substrate. The reaction velocity with DL-dopa was nearly identical to pyrogallol when given the same 5 min of incubation. For pyrogallol, we found a substantial levels of auto-oxidation at 5 min of incubation, with significant increase throughout the incubation period. Also, the OD measured for oxidation of the substrate by serum PO was found to decrease gradually unlike other substrates (fig. 6). This was again due to abrupt auto-oxidation of the substrate that interfered in the measurement of original oxidation of the substrate by the serum PO. For these reasons, pyrogallol was not an option, so DL-dopa (fig. 7) was chosen as the appropriate phenolic substrate of serum PO in the grub of *O. rhinoceros* to carry out further assays of PO activity.

Effect of substrate concentration on serum PO activity

To determine the optimal concentration, the serum PO activity was assayed with different concentrations of DL-dopa (5, 7.5, 10, 12.5 and 15 mM), the level of oxidation gradually increased with increase in substrate concentration and reached the highest activity at the concentration of 10 mM of DL-dopa (fig. 8).

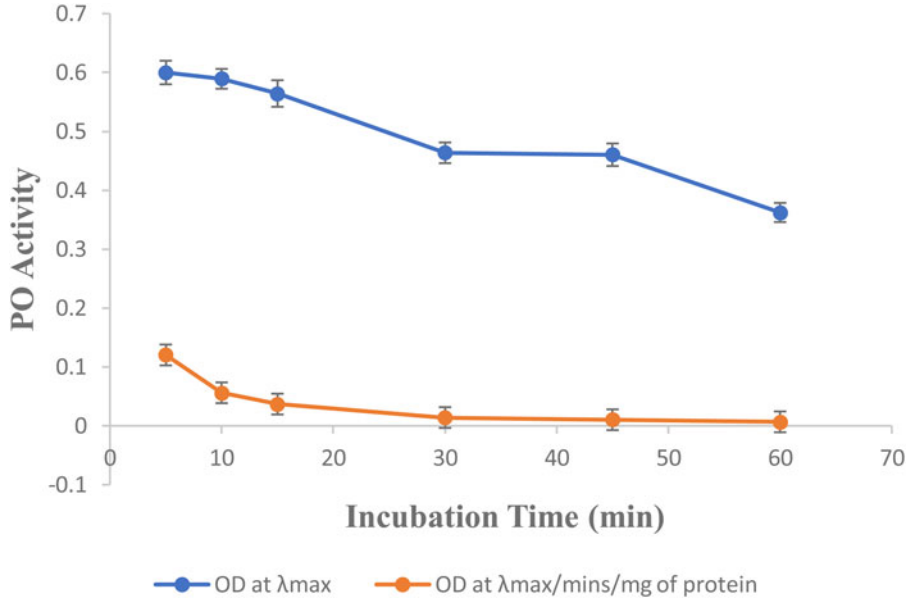

Figure 6. Time course of action of substrate pyrogallol on serum phenolic activity in the grub of *O. rhinoceros*. Values represent mean ± SD of three determinations.

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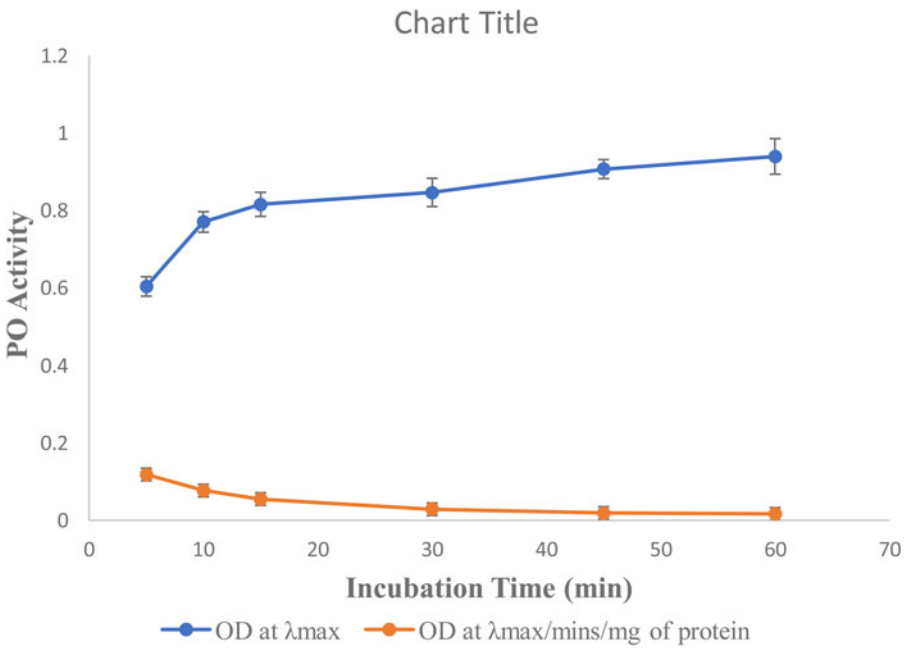

Figure 7. Time course of action of substrate DL-dopa on serum phenolic activity activity in the grub of *O. rhinoceros.* Values represent mean ± SD of 3 determinations.

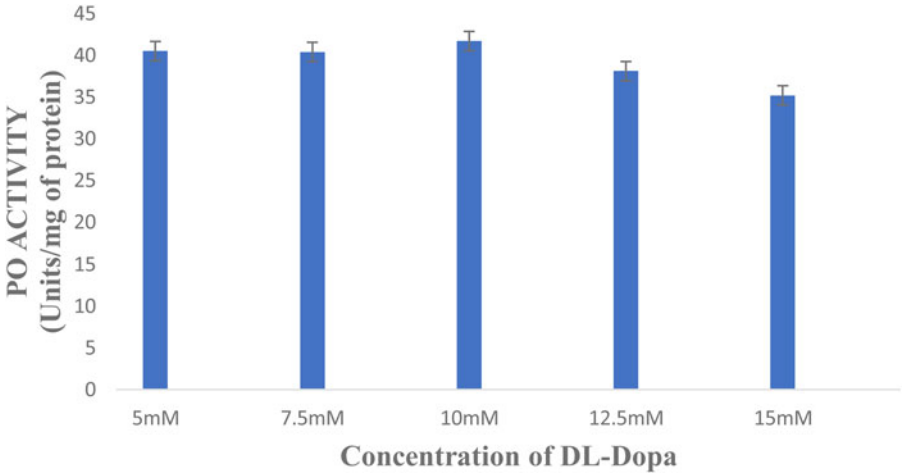

Figure 8. Effect of various concentrations of DL-dopa on oxidation of PO activity in the grub serum of *O. rhinoceros*. Values represent mean ± SD of three determinations.

Thereafter, the reaction velocity dropped with 12.5 and 15 mM concentration of the substrate and hence a range in 5–10 mM DL-dopa was considered to be optimum to perform subsequent PO enzymatic characterization assays in the grub serum of *O. rhinoceros.*

Effect of pH on serum PO activity

To determine the optimal pH, the ability of the serum to oxidize DL-dopa at different potential of hydrogen ion concentration (pH) was examined by using its desirable 10 mM DL-dopa substrate solutions prepared at various pH (6.0, 6.5, 7.0, 7.5, 8.0 and 9.0). Under these conditions, PO exhibited maximum activity at pH 7.5 and decreased at pH 8 and pH 9 (fig. 9).

Effect of temperature on serum PO activity

To determine the optimum temperature, PO activity was examined at different temperatures starting from 10 to 60°C, using 10 mM DL-dopa as the substrate. Under these conditions, PO exhibited maximum activity at the temperature of 20°C. When the serum samples were subjected to 50 and 60°C, a complete precipitation of the serum was observed and thereby indicating denaturation of the proteins. This inferred that PO from the grub serum of *O. rhinoceros* denatured at temperature above 40°C (table 2).

Discussion

In insects, the enzyme phenoloxidase (PO) is extensively reported to play significant role in cuticular sclerotization and defensive functions (Brunet, 1980; Jeyaraj *et al.*, 1986; Andersen, 2010). It often remains in a zymogen form called prophenoloxidase (proPO) and is readily activated by a series of serine protease enzymes to PO by both endogenous and exogenous activating systems (Sugumaran *et al.*, 2000). Even a very minute amount of fungal or bacterial cell wall components are enough to trigger activation of proPO cascade (Ashida and Yamazaki, 1990) and

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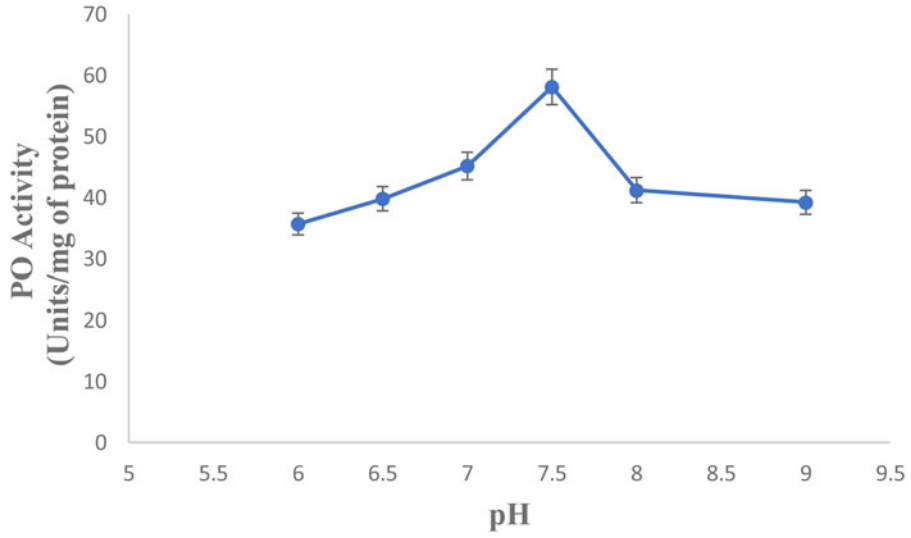

Figure 9. Effect of various pH of DL-dopa on oxidation of PO activity in the grub serum of *O. rhinoceros*. Values represent mean ± SD of three determinations.

Table 2. Effect of various temperatures on oxidation of the substrate DL-dopa by the serum of *O. rhinoceros.*

Temperature (°C)	Phenoloxidase activity (Units mg^{-1})
10	55 ± 0.3
20	63 ± 0.3
30	58 ± 0.4
40	57 ± 0.5
50	-
60	-

Values are shown as mean ± SD of three determinations.
'-' indicates termination of PO activity.

both these microbes are omnipresent, thereby supporting the fact of spontaneous melanization of the haemolymph within a few minutes of collection. PO cascade are involved in initial rapid detection of foreignness and gets activated immediately thereby take part in early defence against non-self in the insect system when compared to other immunoproteins that are synthesized only after injury or invasion of foreign bodies (Ratcliffe *et al.*, 1985). The autoimmune reactions against damaged or aberrant tissues are also reported to trigger proPO cascade in *Drosophila* (Ashida and Yamazaki, 1990). Besides, phenoloxidase activation appears under the constitutive immune response of the innate immune system of insects (Schmid-Hempel, 2005). These types of immune responses are always present at maximum, non-specific in nature and get rapidly activated to eliminate the invading pathogens that are microbial in nature (Laughton and Siva-Jothy, 2011). In addition, it has been reported that spontaneous activation of proPO occurs even at the time of haemolymph collection (Bidla *et al.*, 2009).

All of these data demonstrate that spontaneous haemolymph melanization is inevitable and is caused by the natural conversion of proPO to PO, which is involved in the oxidation of several naturally occurring phenolic substrates in serum and haemolymph. Since PTU, an inhibitor of the enzyme PO and anticoagulants are not used in the haemolymph that is taken for PO investigation, it becomes the main obstacle in understanding PO functions (Ryazanova *et al.*, 2012). It was also revealed that the rate of melanization varied from insect to insect. Thus, in the present study, spontaneous melanization and the rate of melanization at various time intervals were examined and the optimal reaction sample was chosen for the PO assay investigation, as previously stated. The intensity of colour formed due to melanization was much higher in haemolymph when compared to other four samples. This demonstrated that even while the molecules from haemocytes are present in both the haemolymph and the serum, proPO activating factors are somehow present in the haemolymph but not much in the serum. This is also for the reason that the amount of coagulant formed in the haemolymph varies depending on the grub from which it was collected. Additionally, the colour intensity formed in diluted serum A and B was the same, indicating that calcium's presence or absence has no bearing on the melanization process in the samples of diluted serum. Based on these observations, crude serum was chosen to measure PO activity because melanization happens at a moderate rate, which is ideal for PO assays.

Furthermore, as PO is an enzyme, standardising its preferred phenolic substrate is a necessary step and has a significant impact on the thorough investigation of serum PO in any insect system. Nevertheless, the majority of PO substrates have a propensity to spontaneously oxidize and produce falsely positive optical density measurements, which must be taken into consideration. The gap in the most of earlier investigations is that researchers frequently choose one phenolic substrate and one activator to activate PO enzyme before moving forward with PO spectroscopic experiments for characterising and purifying this enzyme (Chase *et al.*, 2000; Rafiei *et al.*, 2018; Baruah *et al.*, 2019; Yousefi-Lardeh and Zibaee, 2020). Despite the fact that this is possible, standardising preferred phenolic substrates for the isolating PO enzyme is an advantage and improves the work's suitability, certainty and species specificity. Therefore, a thorough screening of substrate suited for serum PO is crucial to comprehend the immunological response of insects.

A total of eight substrates classified under monophenols (tyrosine and tyramine), diphenols (dopamine, L-dopa, DL-dopa, catechol and protocatechuic acid) and polyphenol (pyrogallol) were chosen and the absorption maxima respective to each substrate was methodized (Lee *et al.*, 2000; Asano and Ashida, 2001; Benesova *et al.*, 2009; Valadez-Lira *et al.*, 2012; Sharifi *et al.*, 2015; Arumugam *et al.*, 2017; Baruah *et al.*, 2019). It is due to the kind of phenoloxidase present in the serum of a particular

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insect (monophenol oxidase, diphenol oxidase, or polyphenol oxidase) tends to affect the absorption maxima specific to each substrate. This is demonstrated in various research works where oxidation of the same substrate, such as L-dopa was measured at several absorption maxima (Da Silva *et al.*, 2000; Gholami *et al.*, 2013; Clark, 2015). Additionally, measurements at a wavelength where each substrate absorbs the most ensure that the analysis is sensitive to the range of substrate concentrations that are provided.

The affinity of the serum PO enzyme among the different phenolic substrates tested at its respective absorption maxima was found to be highest for the diphenolic substrate, DL-dopa. Several research studies were also used DL-dopa as most predominant phenolic substrate to study haemolymph PO activity (Hung and Boucias, 1996; Beck *et al.*, 2000; González-Santoyo and Córdoba-Aguilar, 2012; Valadez-Lira *et al.*, 2012). Further, optimum concentration of DL-dopa, to be used for PO spectroscopic assay was also devised by subjecting the PO assays with various concentrations of the substrates. It was found that 10 mM DL-dopa as optimum and desirable concentration to carry out PO assays. Moreover, 10 mM concentration of DL-dopa is used for carrying out PO assays in the haemolymph of several insects like *Heliothis virescens, Plodia interpunctella, Spodoptera exigua* and *Trichoplusia ni* (Shelby and Popham, 2008; Valadez-Lira *et al.*, 2012).

Studies on time course of oxidation of phenolic substrates by serum PO confirmed that prolonged incubation time with the substrate can eventually reduce the concentration of serum PO available for the assay. This is evident by the reduction of rate of the reaction velocity with the substrate upon increasing time of incubation. On the contrary, the reaction velocity is maximum for minimal incubation period of 5 min and the same incubation period is used to study PO activity in several of the previous works (Ajamhassani *et al.*, 2012; Zdybicka-Barabas *et al.*, 2014). These findings substantiated that 5 min to 10 min is the optimum period of incubation for carrying PO assays in *O. rhinoceros*. Further, the proenzyme nature of PO in the insect system could be attributed to prevent the self-deleterious effects of PO (Nappi *et al.*, 2005) and the cytotoxic intermediates that are formed during activation of PO cascade, as these could react with reactive oxygen species which are formed as natural by-products of normal body metabolism (Saul and Sugumaran, 1987). In addition, activation of PO cascade is a tightly controlled process and PO activating enzymes present in the haemolymph neutralize the excess activated PO over a period of time to combat the pleiotropic effects of PO (Cerenius *et al.*, 2008). This is also one of the major reasons to circumvent longer incubation period of serum PO with the substrate (Schmid *et al.*, 2008) as PO activating enzymes might interfere in the original activity. Also, less incubation period of 5 min for studying haemolymph PO activity in insects was previously reported in several studies (Halwani *et al.*, 2000; Asano and Ashida, 2001; Kim *et al.*, 2002; Bae and Kim, 2004; Kamalanathan *et al.*, 2020).

Temperature and pH are the two important parameters that affect the activity of an enzyme reaction. Under various physiological circumstances, sudden changes in these variables could have considerable impact on PO activity leading to alter immune responses. The serum PO activity of *O. rhinoceros* remains stable up to a temperature of 40°C (Sadawarte *et al.*, 2019) and shows optimum activity at a lesser temperature of 20°C. This low temperature preference is due to the fact of habitat preference of the larva which usually digs itself into the moist cow dung manure where the temperature in general prevails around 20 to 30°C. Further, complete precipitation of haemolymph was observed on incubation at 50 and 60°C indicating abrupt inactivation of PO at these elevated temperatures due to protein denaturation (Ashida, 1971; Tsukamoto *et al.*, 1986; Ajamhassani *et al.*, 2012). The obtained results were in concordance with optimal temperature of PO activity reported in *Apis mellifera* (Zufelato *et al.*, 2004) and *Helicoverpa armigera* (Sadawarte *et al.*, 2019) and were lower to optimal temperature of PO activity reported in *E. integriceps* (35°C) (Zibaee *et al.*, 2011) and *Antheraea assamensis* (37°C) (Baruah *et al.*, 2019). The optimum pH for serum PO activity was found to be a nearly neutral *i.e.*, 7.0–7.5 and the results were in concordance with the optimal pH reported in *Bombyx mori* (Ashida, 1971), *Ostrinia furnacalis* (Feng *et al.*, 2008), *Apis mellifera* (Zufelato *et al.*, 2004), *Pieries rapae* (Xue *et al.*, 2006) and *Antheraea assamensis* (Baruah *et al.*, 2019).

On the whole, this study has made a serious attempt to define a suitable protocol to standardize a specific substrate for studying circulatory phenoloxidase present in any insect system. It is by considering various parameters from validating spontaneous melanization of the collected haemolymph to the time of incubation for the oxidation of the substrate by the enzyme as these are necessary prerequisites which are not at all disregarded.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485323000305.

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