International Journal of Agricultural Technology 2014 Vol. 10(5):1101-1113
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

In Vitro Signal Transduction to Trigger and Suppress the Phenoloxidase Cascade of Fourth Instar Larvae of *Ariadne Merione* (Cramer)

Sivaprasath, P. and Nalini, M.*

Department of Biotechnology, Karpagam University, Coimbatore 641021, India.

Sivaprasath, P. and Nalini, M. (2014). *In vitro* signal transduction to trigger and suppress the phenoloxidase cascade of fourth instar larvae of *Ariadne merione* (Cramer). International Journal of Agricultural Technology 10(5):1101-1113.

Abstract Activation of Phenoloxidase (PO) was the main frontline defense mechanisms in arthropods. The non-self molecules are determined by pattern recognition (cell wall compositions) and immediately encountered. Phenoloxidase activity was detected in the hemolymph fractions (Serum, Plasma, Hemocyte lysate solution (HLS)) of *Ariadne merione* and pro-enzymes were activated *in vitro* using proteases (Trypsin and α-chymotrypsin), detergents (Triton X-100 and Sodium dodecyl sulphate) and laminarin (polymer of β-1,3-glucan). α-chymotrypsin and laminarin activated the PO cascade at higher level comparing with other activators. PO level in hemolymph fraction of *Ariadne merione* responded to a maximum extent on pre incubating the test samples with bacteria (*salmonella paratyphi, Klebsiella pneumoniae, Staphylococcus typhi, Streptococcus epidermis, Pseudomonas aeruginosa, Proteus* sp., *Staphylococcus aureus* and *Escherichia coli*). The PO activating cascade was interfered and suppressed by introducing certain inhibitors (phenylthiourea (PTU), dithiothreitol (DTT), dexamethasone and L-tyrosine) into the test samples. PTU showed the maximum inhibition rate in all test samples (hemolymph fractions). The impact of calcium was also monitored by pre-incubating the samples with TBS buffer containing calcium chloride from 0.1 mM to 10 mM concentration. As the concentration increased the PO activation was increased this primary data proves the essential of calcium in host immune system of *Ariadne merione*.

Keywords: Phenoloxidase (PO), *Ariadne merione*, Serum, Plasma, Hemocyte lysate solution (HLS)

Introduction

The prophenoloxidase-activating system is one of the main defense mechanism in arthropods in which non-self molecules (bacteria, virus and fungus etc.,) were recognized after invading into the system. Insect phenoloxidase (PO) generates quinones and other reactive intermediates to immobilize and kill invading pathogens and parasites. Due to presumed

* **Corresponding author:** Padmanabhan, M. N.**: E-mail:** madanagopalnalini@yahoo.com

cytotoxicity of these compounds, PO activity and its proteolytic activation have to be regulated as a local transient reaction against non-self in order to minimize damage to the host tissues and cells (Zhiqianglu and Haobojiang, 2007). Defense system of invertebrates is based on both cellular and humoral immune responses. It is known that, humoral immune response is triggered by bacterial carbohydrate components such as lipopolysaccharides (LPS) and β-1,3-glucans. Recognition proteins are unable to destroy foreign matter, and are related to other defense system responses (Cheng *et al.,* 2005). Melanization is an important immune mechanism in arthropods and possibly among many other invertebrate taxa, been less investigated later. This innate immune reaction provides toxic quinone substances and other short-lived reaction intermediates. Recent evidence also strongly implies that the melanization (prophenoloxidase activating) cascade is intimately associated with the appearance of factors stimulating cellular defense by aiding phagocytosis and encapsulation reactions (Cerenius *et al.,* 2008).

In addition, tissue damage inflicted by mechanical wounding and possibly by enzymes released from pathogens will also lead to proPO activation (Galko and Krasnow, 2004). Several critical steps including proteolytically clearing proPO into active PO in the melanization cascade are carried out by serine proteinases. Therefore, proteinase inhibitors such as serpins (serin proteinase inhibitors) are involved in preventing premature and excessive activation (Cerenius *et al.,* 2008). In crayfish, *Pacifastaeus leniusculus*, and probably in other crustaceans as well, a large molecular weight inhibitor, pacifastin (a unique heterodimeric protein containing a transferrin and an inhibitor chain) acts as a potent inhibitor of terminal serine proteinase in activation cascade (Liang *et al.,* 1997). Activation of proPO in insects is probably mediated by a serine proteinase cascade, analogous to the coagulation pathway and complement system in human plasma (Sugumaran, 1996; Sugumaran and Kanost, 1993; Gillespie *et al.,* 1991). Components of this proteinase system in insects may be already present in circulating hemolymph or released from hemocytes or fat body when pathogens or parasites are encountered. Recognition of invading microorganisms or aberrant host tissue may trigger the auto activation of the first proteinase in the pathway, which leads to the sequential activation of their components in the system through limited proteolysis (Jiang *et al.,* 2003). Pro-phenoloxidase activating proteinase (PAP) (also known as phenoloxidase – activating enzyme (PAPE)) is the terminal enzyme that directly converts proPO to PO. The reactions catalyzed by phenoloxidase can then result in melanization of foreign organisms trapped in capsules or hemocyte nodules (Gillespie *et al.,* 1991).

Antimicrobial effect of reactive intermediates was produced in phenoloxidase–catalyzed reactions. After being treated with *Manduca sexta* phenoloxidase and dopamine, *Escherichia coli* and *Bacillus subtilis* ceased to grow, whereas the growth of *Pichia pastoris* was slightly affected (Zhao *et al.*, 2007). Po generates reactive compounds such as 5,6-dihydroxyindale (DH1), which have a broad-spectrum antibacterial and antifungal activity. DH1 reports that spontaneous oxidation products are also active against viruses and parasitic wasps (Zhao *et al.,* 2011).

Phenoloxidase is a key enzyme responsible for the catalysis of the melanization reaction. Fuel product melanin is induced in wound healing and immune responses. Prophenoloxidase cascade has been widely described in arthropods (Prochazkova *et al.,* 2006).

In this study, the level of signal transduction of prophenoloxidase system of *A. merione* hemolymph fractions triggered by non self molecules (bacteria) and chemical components like trypsin, α-chymotrypsin, triton X100, SDS and laminarin. The level of inhibition also studied using different type of inhibitor like phenylthiourea (PTU), dithiothreitol (DTT), dexamethasome and L-tyrosine. Also the effect of calcium on proPO activation was determined using different concentration of calcium chloride.

Materials and methods

Experimental animal

Ariadne merione (first and second instars) larvae were collected in and around Kuruchi area (Latitude10.9600 °N, Longitude 77.0100 °E), Coimbatore, India. The larvae were maintained under laboratory conditions provided with proper diet. Fourth instar larvae were used for the experiment.

Hemolymph collection

Hemolymph was collected by cutting the pro leg of the larva. Oozying hemolymph was collected in eppendroff tube held on ice and centrifuged at 400 x *g* for 10 minutes. Followed by that hemolymph fraction serum, plasma, hemocytes lysate solution (HLS) were prepared.

Assay of PO activity

Prepared hemolymph fractions (serum, plasma and HLS) test sample 50 µl was taken in 5 ml test tube and 50 µl TBS buffer (250 mM, pH 6.5) was added and pre-incubated at room temperature for 15 minutes. After 15 minutes,

1ml of substrate L-1,3,-dihydroxyphenylalanine (DOPA) was added to the tube and incubated kept for 15 minutes at room temperature and OD read at 450 nm.

Effect of activators on proPO levels

The corresponding test samples 50 µl were pre-incubated with 50 µl activators like, Trypsin, α-chymotrypsin, triton X100, SDS and laminarin (mg/ml concentration) at room temperature for 15 minutes followed by 1 ml substrate L-DOPA was added and again incubated at room temperature for 15 minutes after that the mixture was read at 450 nm.

Effect of inhibitors on proPO levels

A 50 µl test samples was treated with 50 µl inhibitors Phenylthiourea (PTU), Dithiothreitol (DTT), Dexamethasone, and L-Tyrosine (mg/ml concentration) were incubated at room temperature for 15 minutes after 1 ml substrate L-DOPA was added and again incubated at room temperature for 15 minutes. Finally the OD was read at 450 nm.

Level of proPO against exogenous molecules

Bacterial suspension was prepared using 8 bacterial cultures, consists a series of Gram positive and negative bacteria includes *Salmonella paratyphi, Klebsiella pneumoniae, Staphylococcus typhi, Streptococcus epidermis, Pseudomonas aeruginosa, Proteus* sp., *Staphylococcus aureus* and *Escherichia coli*. A 50 µl test samples were pre-treated with 50 µl of bacterial cultures and incubated for 15 minutes after that 1ml substrate (DOPA) was added and again incubated for 15 minutes at room temperature and finally read at 450 nm.

Effect of Calcium chloride on proPO activation

Effect of calcium on proPO activation was studied by pre-incubating the test samples at room temperature for 15 minutes in TBS buffer containing $CaCl_2$ in different concentrations from 0.1mM to 10 mM followed by addition of 1ml substrate (DOPA) and incubated for 15 minutes at room temperature and finally read at 450 nm.

Results

The oxidation reaction of L-DOPA by the hemolymph fractions (serum, plasma and HLS) of *Ariadne merione* were explained by triggering the proPo system using proteases trypsin, α-chymotrypsin, and detergents triton X100,

SDS and polymer (β-1,3-glucan) laminarin. α-chymotrypsin showed increased levels, in serum (PO > 0.0027), plasma (PO > 0.0026) and in HLS (PO > 0.0090) PO activity (units. min^{-1}. mg $protein^{-1}$) shown in Fig 1.

Inhibitors used to interfere with oxidation of L-DOPA, phenylthiourea (PTU), dithiothreitol (DTT), dexamethasone, and L-tyrosine was used as inhibitors. PTU showed significantly highest level of inhibition, in serum (PO < 0.0017), plasma (PO < 0.0011) and in HLS (PO < 0.00141) PO activity (units. min^{-1}. mg $protein^{-1}$) when compared to control value. The levels of all inhibitors were displayed in Fig 2.

Signal transduction of activating PO was also archived by exogenous molecules (bacteria), there was a great response for the non-self molecules. Bacteria includes *Salmonella paratyphi, Klebsiella pneumoniae, Staphylococcus typhi, Streptococcus epidermis, Pseudomonas aeruginosa, Proteus* sp., *Staphylococcus aureus* and *Escherichia coli* were used for determine the level of PO activation. *Staphylococcus paratyphi* level of activation was increased in serum (PO > 0.0025), plasma (PO > 0.0036) and in HLS (PO > 0.00143) PO activity (units. min^{-1}. mg $protein^{-1}$) showed in Fig 3.

Effect of calcium on proPo activation was also studied by increasing the calcium chloride concentration in TBS buffer used in the assay. PO level was increased under the influence of calcium in serum, for 0.1 mM (PO > 0.0032), 0.5 mM (PO > 0.0034), 0.7(PO > 0.0035), 1 mM (PO > 0.0038), 3 mM (PO > 0.0040), 5 mM (PO > 0.0042) and 10 mM (PO > 0.0043) PO activity (units. min^{-1}. mg $protein^{-1}$). Gradual increase of PO activity was displayed in Table 1.

Table 1. Effect of $CaCl_2$ on hemolymph fraction (Serum, Plasma and HLS) of *A. merione* at different concentrations. The table shows rate of L-DOPA oxidation and results in gradual increase of PO level

S. No	$CaCl_2$ concentration (mM)	PO units/min/mg protein in test samples*		
		Serum	Plasma	HLS
1	control	0.0031 ±0.0002	0.0022 ±0.0003	0.0178 ±0.0010
2	0.1	0.0032 ±0.0003	0.0028 ±0.0002	0.0196 ±0.0013
3	0.3	0.0034 ±0.0005	0.0032 ±0.0001	0.0201 ±0.0009
4	0.7	0.0035 ±0.0002	0.0035 ±0.0001	0.0210 ±0.0010
5	1	0.0038 ±0.0001	0.0038 ±0.0003	0.0223 ±0.0008
6	3	0.0040 ±0.0002	0.0043 ±0.0005	0.0227 ±0.0013
7	5	0.0042 ±0.0002	0.0044 ±0.0005	0.0235 ±0.0010
8	10	0.0043 ±0.0005	0.0049 ±0.0003	0.0247 ±0.0015

*Values are given as mean ±SD of three determinations.

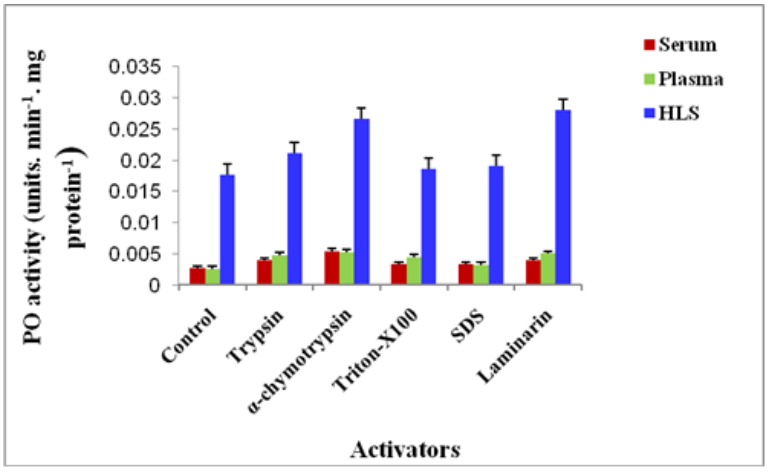


Fig. 1. Effect of exogenous proteases, detergents and non-self molecule on PO activity in hemolymph of IV instar of *A. merione*. Phenoloxidase activation rate in the hemolymph fractions increased in presence of activators the rate of L-DOPA oxidation was increased on signal transduction sent to the proPO activating cascade.

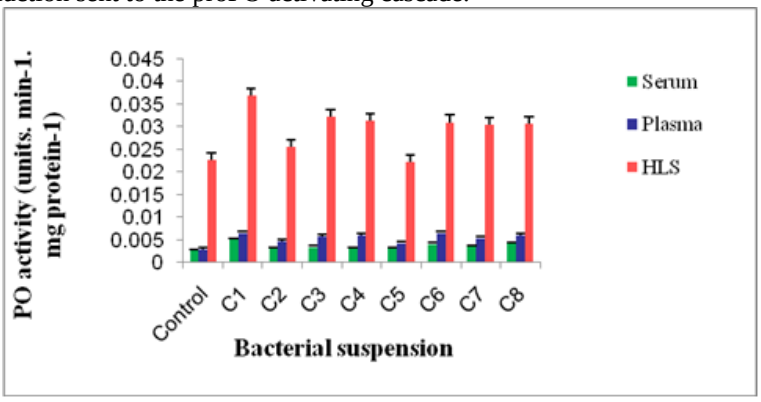


Fig. 2. PO system triggered after introducing bacterial suspensions *in vitro* in the hemolymph fractions of *A. merione*. Bacterial suspension used in activating PO cascade was C1-*salmonella paratyphi*, C2-*Klebsiella pneumoniae*, C3-*Staphylococcus typhi*, C4-*Streptococcus epidermis*, C5-*Pseudomonas aeruginosa*, C6-*Proteus* sp., C7-*Staphylococcus aureus* and C8-*Escherichia coli*.

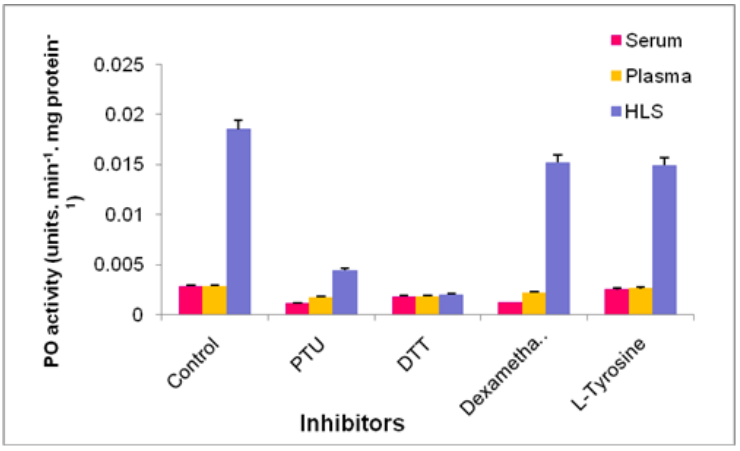
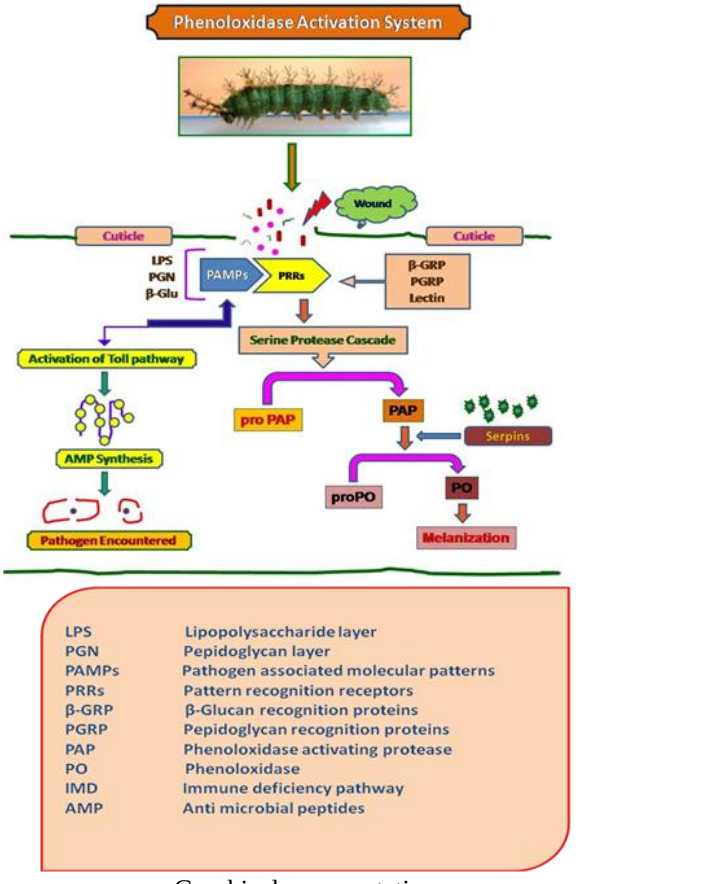


Fig. 3. Effect of protease inhibitors on phenoloxidase activity in hemolymph of IV instar of *A. merione*.



Graphical representation

Discussions

In the present study, the signal transduction of proPo system into active form by the activators, inhibitors and exogenous molecules were analyzed through spectrophotometer. The oxidation of L-DOPA by the hemolymph fractions, i.e. the serum plasma (HLS) of *A. merione* was clearly explained that molecules involved in triggering the proPO system are proteases trypsin, α-chymotrypsin, and detergents like, triton X100, SDS and Laminarin.

The activation compared with the control values of proPO activators. The rate of activation was seen in HLS. The presence of proPO in hemolymph fractions was shown in *Perna viridis*, compared with plasma and hemolymph, hemocytes containing 20 folds more proPO activated in *P. viridis* (Asokan *et al.,* 1997). Researchers also reported the up regulation of PO level till 21 days in the system of *Daphnia mayna* after the infection made with *Pasteuria ramose* (Pauwels *et al.,* 2011). The proPO mechanism was explained in Eisenia fetida with various substrates (L-DOPA, dopamine, N-acetyl-dopamine, 4-methyl catechol and tyrosin) the author conclude the earth worm PO activity is lower than in Arthropods (Prochazlcora *et al*., 2006).

The activation of Po level in serum and plasma was shown in Fig 1. They are relatively same in activation and viewing the HLS activation is greatly higher than serum and plasma. Analyzing the activators use of the proteases shows higher activity value i.e., PO activity (units. min^{-1}. mg $protein^{-1}$), α-chymotrypsin shows the higher activation comparing with other protease and detergent next to the α-chymotrypsin the proPo cascade was well activated by laminarin activated the level of PO in HLS than the α-chymotrypsin.

Laminarin was used to trigger the proPO cascade in *Manduca sexta* for extraction and purification of GRP (β-1,3-Glucan recognition protein) from the hemolymph. This helps in understanding the immune system, binding specificity of the immune molecules in invertebrates (Jiang *et al.,* 2004). The activation of PO was demonstrated in mosquito species after adding laminarin and trypsin that increases the PO activity (Nayar and Bradely, 1994).

The proPO activation also studied by inhibiting the cascade, the level of inhibition was clearly monitored using certain inhibitors. Inhibitors like phenylthiourea (PTU), dithiotreital (DTT), dexamethasome and L-lysine. The inhibition action was seen at greater level by using the phenylthiourea, the rate of inhibition was recorded at spectrometrically. Compared to the control PO activation was inhibited up to 7 folds. Next to the phenylthiourea the inhibition rate was recorded in dithiotreitol the rate of action was inhibited up to 4 folds comparing with the control. The activity of PTU was demonstrated in mosquito species *Aedes aegypti* and *Anopheles quadrimaculatus* the Po activation was reduced comparing with the buffer control (Nayar and Bradely, 1994).

The effect of PTU on oxidation of L-DOPA by HLS and plasma was clearly studied in *Perna viridis* the PO activity was inhibited up to 50 % in plasma and 12 % in HLS by the PTU activity (Asokan *et al.,* 1997). In other hand the inhibition rate was recorded in least values by using the dexamethosome and L-tyrosine.

Analyzing these inhibition activity of certain compounds on arthropods and other invertebrates helps us to study the immune strengths of the animal and it helps in developing new compounds to eradicate the pests which attacking on food crops. Certain virus, bacteria and fungus produces the serine protease cascade and phenoloxidase activity proteases for survival in the specific host. Monitoring and studying the nature of the specific microbe toxic molecules and its molecular dynamics properties which interacts and inactivate the immune systems of the hosts. They will help in designing new insecticides and pesticide which is of less toxic to the environment.

Pretreatment with laminarin PO activity was enhanced in both plasma and HLS of *Perna viridis* that indicates the activation of proPO in the hemolymph function triggered by non-self molecules (Asokan *et al.,* 1997). The proPO activation was also recorded in *Ariadne merione* after treating the hemolymph fractions with eight types of bacteria was utilized and those responses were increased for all the bacterial cultures when comparing with control.

More response to the gram-positive bacteria comparing with the gram-negative, *salmonella paratyphi* triggered the larval of phenoloxidase (PO) at high level. The lowest PO activity was observed at *Pseudomonas aeruginosa* and *Klebsiella phenumoniae*. Constantly the PO level was same remaining pathogens like *Staphylococcus typhi*, *Streptococcus epidermis*, *Proteus* sp., *Staphyloccus aureus* and *Escherichia coli*. Lipopolysaccharide obtained from *E. coli* and *Pseudomonas auruginosa* enhanced the PO activity in *Perna viridis* (Asokan *et al.,* 1997). *Spodoptera exigua* larvae was infected using *Beauveria bassiana* blastospores and observed the PO activation enhanced in the hemolymph, plasma samples from 24 hours. Post challenged larvae should significant increase in PO level up to five fold (Hung and Boucias, 1996).

On the other hand, analyzed the immune defense of these arthropods will provide idea for triggering new type of antimicrobial peptides for different pathogens. It may useful for human to get rid of certain bacterial and viral diseases. An invertebrate produces a novel antibacterial or antiviral protein according to the membrane chemical nature. These protocol will help to get new antibacterial protein in invertebrate and tracking the gene responsible and cloning the gene in a bacteria and producing the antibacterial molecules for pharmaceutical utilization against diseases. An antibacterial peptide was isolated from the hemolymph of *Rhodaius pralixus*. The isolated peptide belong

to the defensin family, also the cDNA clones obtained from RNA extracted from whole body of *Rhodnius prolixus* (Lopez *et al.,* 2003). Antimicrobial peptides was induced against gram positive and gram negative bacteria in the IZD-MB-0503 cell line derived immunocytes of the insect *Mamestra brassicae* (Mandrioli *et al.,* 2003).

Novel group of peptide with varying chemical and molecular dynamic properties that can be analyzed and isolated from vertebrates and invertebrates resulting in new generation for antimicrobial peptides and it can be endogenously expressed in genetically modified organisms (Marshall and Arenas, 2003). A novel homodimeric antimicrobial peptide was purified from the hemocyte of *Halocynthia aorantium*. It has broad-spectrum activity against gram positive and gram negative bacteria (Lee *et al.,* 2001). An antimicrobial peptide belongs to the Cecropin family was isolated from the hemolymph of *Aedes aegypti* (Lowenberger *et al.,* 1999).

cDNA encoding phenoloxidase in the hemocytes of *spodoptera litura* was expressed in *E. coli* and recombinant peptide was isolated the protein is catalytically active on 4-Methyl pyrocatechol upon activation by acetyl pyridinium chloride (Rajagopal *et al.,* 2005). An antibacterial peptides consisting of 26 amino acid residue was found in the plasma of fresh water crayfish and it has broad-spectrum against gram positive and gram negative bacteria (Lee *et al.,* 2003). A serine proteinase cascade was proved in *Manduca sexta*. Isolation of cDNA encodes for HP14 proteinase, which triggers the cascade during invasion of foreign bodies into the system (Ji *et al.,* 2004).

Phenoloxidase existed as a zymogen which could be activated by chymotrypsin was studied in 5 species of grass hoppers (Michael *et al.,* 1989). The level of phenoloxidase activity was shown in different stages (egg, larvae and pupa) of cotton worm *Spodoptera littoralis*. Find conclusion was given i.e., there are possibilities of a hormonal mechanism directing the system (Ishaaya, 1972; Ishaaya and Navon, 1974), towards bacterial challenging response. Another type of phenol activating proteinase PAP-2 was isolated from the integuments of *Manduca sexta* (Jiang *et al.,* 2003). Evolutionary dynamics of immune system comparison with insect immune transcriptomes was studied using the exogenous and endogenous triggering of their respective immune system (Bidla *et al.,* 2008).

Calcium play an important role in the immune system of *Ariadne merione* chelation of cations in the hemolymph ultimately it loses the agglutination reaction (Unpublished data). A particular study was due to know the impact of $CaCl_2$ on the hemolymph. Surprisingly the PO activation rate was activated and started to enhanced level by level on using different concentration of $CaCl_2$ from 0.1mM to 10 mM was used. The $CaCl_2$ required was very low in *Perna*

viridis for maximum activation of hemolymph fractions (plasma and hemocyte) proPO by non-self molecules (Asokan *et al.*, 1997). Also there are some reports i.e., calcium was not required for activation of PO in the hemolymph of *Aedes aegypti* and *Aedes auadrimaculatus* (Nayar and Bradley, 1994).

Insect innate immunity consists of both cellular and humoral responses that functionally associated with each other during immune response. They recognize the type of pathogens/parasites, toxicity level, route of infection and physiological state may differs but the efficacies of individual responses will be a greater level. Our study implied a critical role of proPO activation and responses. Further investigations are required to better understand the biochemical functions of serine proteinases cascade in insect immunity. Future work will be concentrated on antimicrobial peptides and evolving these peptides to a successful pharmacy product against infectious human pathogens.

References

Asokan, R., Arumugam, M. and Mullainadhan, P. (1997). Activation of prophenoloxidase in the plasma and hemocytes of the marine mussel *perna viridis* linnaeus. Developmental and Comparative Immunology 21:1-12.

Bidla, G., Hauling, T., Dushay, M.S. and Theopold, U. (2008). Activation of insect phenoloxidase after injury: endogenous versus foreign elicitors. Journal of Invertebrate Immunology 1:301-308.

Cerenius, L., Lee, B. L. and Söderhäll, L. (2008). The proPO-system: pros and cons for its role in invertebrate immunity. Cell Press 29:263-271.

Cheng, W., Liub, C. H., Tsaia, C. H. and Chen, J. C. (2005). Molecular cloning and characterisation of a pattern recognition molecule, lipopolysaccharide- and β-1,3-glucan binding protein (LGBP) from the white shrimp *Litopenaeus vannamei*. Fish and Shellfish Immunology 18:297-310.

Galko, M. J. and Krasnow, M. A. (2004). Cellular and genetic analysis of wound healing in *drosophila* larvae. PLoS Biology 2:1114-1126.

Gillespie, J. P., Bidochka, M. J. and Khachatourians, G. G. (1991) Separation and characterization of grasshopper hemolymph phenoloxidase by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Comparative Biochemistry and Physiology 98C:351-358.

Hung, S. Y. and Boucias, G. (1996). Phenoloxidase activity in hemolymph of naïve and *Beauveria bassiana* - infected *Spodoptera exigua* larvae. Journal of Invertebrate Pathology 67:35-40.

Ishaaya, I. (1972). Studies of the hemolymph and cuticular phenoloxidase in *spodoptera littoralis* larvae. Insect Biochemistry 2:409-419.

Ishaaya, I. and Navon, A. (1974). Phenoloxidase activity at various stages of development in the egyptian cotton worm *spodoptera littoralis.* Insect biochemistry 4:67-73.

Ji, C., Wang, Y., Guo, X., Hartson, S. and Jiang, H. (2004). A pattern recognition serine proteinase triggers the prophenoloxidase activation cascade in the tobacco hornworm, *manduca sexta.* The Journal of Biological Chemistry 279:34101-34106.

Jiang, H., Ma, C., Lu, Z. Q. and Kanost, M. R. (2004). β-1,3-Glucan recognition protein-2 (β GRP-2) from *Manduca sexta*: an acute-phase protein that binds β -1,3-glucan and lipoteichoic acid to aggregate fungi and bacteria and stimulate proPhenoloxidase activation. Insect Biochemistry and Molecular Biology 34:89-100.

Lee, I. H., Lee, Y. S., Kim, C. H., Kim, C. R., Hong, T., Menzel, L., Boo, L. M., Pohl, J., Sherman, M. A., Waring, A. and Lehrer, R. I. (2001). Dicynthaurin: an antimicrobial peptide from hemocytes of the solitary tunicate, *halocynthia aurantium.* Biochimica et Biophysica Acta 1527:141-148.

Lee, S. Y., Lee, B. L. and Söderhäll, L. (2003). Processing of an antibacterial peptide from hemocyanin of the freshwater crayfish *pacifastacus leniusculus*. The Journal of Biological Chemistry 278:7927-7933.

Liang, Z., Jensen, L. S., Aspán, A., Hall, M. and Söderhäll, K. (1997). Pacifastin, a novel 155-kda heterodimeric proteinase inhibitor containing a unique transferrin chain. Proceedings of the National Academy of Sciences 94:6682-6687.

Lopez, L., Morales, G., Ursic, R., Wolff, M. and Lowenberger, C. (2003). Isolation and characterization of a novel insect defensin from *rhodnius prolixus*, a vector of Chagas disease. Insect Biochemistry and Molecular Biology 33:439-447.

Lowenberger, C., Charlet, M., Viziolii, J., Kamal, S., Richman, A., Christensen, B. M. and Bulet, P. (1999). Antimicrobial activity spectrum, cDNA cloning, and mRNA expression of a newly isolated member of the cecropin family from the mosquito vector *aedes aegypti*. The Journal of Biological Chemistry 274:20092-20097.

Lu, Z. and Jiang, H. (2007). Regulation of phenoloxidase activity by high- and low-molecular-weightinhibitors from the larval hemolymph of *Manduca sexta.* Insect Biochemistry and Molecular Biology 37:478-485.

Mandrioli, M., Bugli, S., Saltini, S., Genedani, S. and Ottaviani, E. (2003). Molecular characterization of a defensin in the IZD-MB-0503 cell line derived from immunocytes of the insect *Mamestra brassicae* (Lepidoptera). Biology of the Cell 95:53-57.

Marshall, S. H. and Arenas, G. (2003). Antimicrobial peptides**:** a natural alternative to chemical antibiotics and a potential for applied biotechnology. Electronic Journal of Biotechnology 6:3-7.

Michael, J. B., Gillespie, J. P. and George, K. G. (1989). Phenoloxidase activity of acridid grasshoppers from the sub families melanoplinae and oedipodinae. Comparative Biochemistry and Physiology 94B:117-124.

Nayar, J. K. and Bradley, T. J. (1994). Comparative study of hemolymph phenoloxidase activity in *aedes aegypti* and *anopheles quadrimaculatus* and its role in encapsulation of *brugia malayi* microfilariae. Comparative Biochemistry and Physiology 109A:929-938.

Pauwels, K., Meester, L. D., Decaestecker, E. and Stoks, R. (2011). Phenoloxidase but not lytic activity reflects resistance a *pasteuria ramosa* in *daphnia magna*against. Biology Letters 7:156-159.

Prochazkova, P., Silerová, M., Stijlemans, B., Dieu, M., Halada, P., Joskova, R., Beschin, A., Baetselier, P.D. and Bilej, M. (2006) Evidence for proteins involved in prophenoloxidase cascade *eisenia fetida* earthworms. Journal of Comparative Physiology B 176:581-587.

Rajagopal, R., Thamilarasi, K., Venkatesh, G. R., Srinivas, P. and Bhatnagar, R. K. (2005). Immune cascade of *spodoptera litura*: cloning, expression, and characterization of inducible prophenol oxidase. Biochemical and Biophysical Research Communications 337:394-400.

Sugumaran, M., (1996). Role of insect cuticle in immunity. New directions in invertebrate immunology. Fair Haven, NJ: SOS Publications. pp. 355-374.

Sugumaran, M.and Kanost, M. (1993). Regulation of insect hemolymph phenoloxidases. Parasites and pathogens. San Diego: Academic Press. pp. 317-342.

Zhao, P., Li, J., Wang, Y. and Jiang (2007). Broad-spectrum antimicrobial activity of the reactive compounds generated in vitro by *manduca sexta* phenoloxidase. Insect Biochemistry and Molecular Biology 37:952-959.

Zhao, P., Lu, Z., Strand, M. R. and Jiang, H. (2011). Antiviral, anti-parasitic, and cytotoxic effects of 5,6-dihydroxyindole (DHI), a reactive compound generated by phenoloxidase during insect immune response. Insect Biochemistry and Molecular Biology 41:645-652.

(Receiced 18 June 2014; accepted 31 August 2014)