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Phenoloxidase activity in hemolymph of naïve and HaNPV infected larvae of *Helicoverpa armigera* (HB), its characterization and inhibition

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Abstract
Helicoverpa armigera nucleo polyhedrosis virus (HaNPV) is the most promising bio-agent. However, as the age of the larvae increases, then susceptibility to HaNPV decreases. This becomes a bottleneck in its effectiveness. Present study deals with the reasons behind this decrease in efficacy. Role of phenoloxidase (PO) in age related resistance, characterization of PO was studied. Phenoloxidase activity was more in cellular fraction of hemolymph in normal larvae of *H. armigera*. Whereas it was high in plasma fraction of virosed larvae. Moreover its magnitude was more in 5th instar larvae as compared to 3rd instar larvae. The Km of PO from virosed larvae decreased in respect to normal larvae, where as the Vmax in virosed larvae increased over that of normal larvae. Dopamine was found to be the best substrate than any other studied. Enzyme PO was optically active at 40^{0}C in normal larvae as well as virosed. The PO from normal and virosed larvae of *H. armigera* showed almost 100 % enzyme activity up to 30^{o}C, which decreased thereafter. Optimum pH for PO activity was found to be 9.0 for normal and virosed *H. armigera* larvae. Amongst all inhibitors of PO tested SDS was found to be the best followed by EGTA >PTU> EDTA > PMSF> DMSO > Garlic extract. Two PO isozymes were present in hemolymph of normal and virosed *H armigera* larvae.

Keywords: *Helicoverpa armigera*, HaNPV, phenoloxidase, hemolymph, dopamine

Introduction
Phenoloxidase (PO) (EC1.14.18.1) or tyrosynase detected both in hemolymph and cuticle of the insect and arthropods, it is responsible for initiating the biosynthesis of melanin pigment (Prota, 1992). In addition to melanization of cuticle, it is used for colour and camouflage. PO is also uniquely associated with three different physiologically important biochemical processes in insect and other arthropods. These are (1) sclerotization of insect cuticle (Anderson *et al*., 1996; Sugumaran, 1998) [4, 39], (2) encapsulation and melanization of foreign organism observed as a defense reaction (Ashida and Brey, 1995, Gillespie *et al*., 1997; Soderhall *et al*., 1990) [37], (3) wound healing (Lai-Fook, 1966; Sugumaran, 1996) [28]. PO is derived from proenzyme prophenoloxidase (pro PO). The activation pro PO is mediated by Ca^{2+} dependent serine proteases cascade which is activated by various microbial cell wall compounds like lipopolysacacharide, laminarin, zymosan etc.
Helicoverpa armigera (Hubner) Hardwick is the most dreaded pest of all the cultivated crops and has attained global importance as alarming pest. It's larvae generally show reduction in susceptibility to baculovirus infection with increase in age (Jayachandran and Chaudhari, 1996, Kalia and Chuadhari, 2001) [24, 25]. Can an insect species susceptible to a pathogen become resistant to more microorganisms? Host specificity observed with many insect pathogens demonstrated that insect species are naturally resistant to these microorganisms (Narayanan, 2004) [33]. Indeed insects that are susceptible to pathogen can show resistance to various entomopathogens and try to resist infection through morphological, behavioral, developmental (like maturation immunity), physiological, nutritional, biochemical and molecular genetic mechanisms etc. Resistances to this infection are achieved with defensive immune system. Insect immune system includes cellular and humoral components. Besides hemocytes the measurement of Phenoloxidase and lysozyme like enzyme activity in the hemolymph has often been used to estimate disease resistance (Rantala *et al*., 2002) [35]. The unique role played by PO in insect physiology and biochemistry demands a serious study of this enzyme. Enzymatic characterization of PO is important in that it provides a better understanding of this enzyme and its involvement in these important reactions is therefore necessary for insect management.
Inhibition of PO *in vivo* results in impairment of encapsulation responses (Brewer and Vinson, 1971) [6] by blocking quinone formation by phenylthiourea an insect's parasite resistance

can be suppressed (Pay.1974). So better understanding of the current knowledge of the insect biochemical, cellular and humeral defenses mechanism parv the way for the proper management of the pests, especially using various biocontrol agents like parasitoids and pathogens after understanding their strategy in bypassing the insect defense.

Material and Methods

Determination of phenoloxidase activity in larvae of different age group

Different instars larvae of H. armigera i.e. 3rd instar as a susceptible and 5th in star as resistant were used to determine the level of phenoloxidase in hemocytes and plasma of hemolymph of H. armigera. The larvae were treated with predetermined HaNPV concentrations as per LC_{50} values for 3rd and 5th instar.

Phenoloxidase activity was determined by using the methodology of Anderson *et al* (1989) [2]. Two hundred µl of hemolymph was withdrawn from the larvae after 0, 24, 48 and 72 hrs of treatment by puncturing at the base of 2nd proleg the hemolymph so withdrawn was collected into 300 µl of neutralized (NH_4) SO, to achieve 40 per cent saturation. The mixture was kept undisturbed for 30min. A portion of this mixture was retained as whole hemolymph and the rest was centrifuged at 10,000 g for 20 min. The pellet was resuspended in 0.01 M sodium phosphate buffer (pH 7.0). The assay was initiated by addition of 100 µl of sample to the reaction mixture (Tris-HCL, pH 7.5 (700µl) and substrate L-DOPA (400µl) and incubated at 30^0C for 3 min. The increase in the absorption at 490 nm was measured at every minute interval on UV spectrophotometer and specific PO activity was determined and expressed in units/mg of protein as per Hung and Boucles (1996) [21], Anderson *et al* (1989) [2].

Characterization of hemolymph phenoloxidase

The hemolymph phenoloxidase enzyme from normal and virosed larvae of H. armigera were characterized by studying some of the biochemical parameters. Microplate assay was exploited in all the characterization experiments as described by Lockey and Ourth (1992) [29] with slight modifications. All the samples were measured in triplicate.

Enzyme Kinetics and Substrate Specificity

Michaelis constant (Km) and Maximal velocity (Vmax) were worked out for the hemolymph phenoloxidase enzyme from normal and virosed larvae of H. armigera by using different concentration of various substrates viz., L- DOPA, DL-DOPA, L-Tyrosine, Dopamine 4-methylbrenzcatecholin. Different molar concentrations of all the above substrates viz., 15, □□□□□□□□□□□□□□and □□□□ □□M was used for this study. Enzyme activity was determined by performing the microplate assay with 50 □l of hemolymph obtained from normal and virosed larvae of H. armigera

Optimum temperature

Fifty micro liter of hemolymph from normal and virosed larvae of *H. armigera* was taken in the 500□l of PCR tube, volume was made up upto 150□l by using 100□l 0.01M phosphate buffer (pH 7.0), followed by 15 min. incubation at different temperatures viz 10,20,30,40,50,60,70 and 80ºC in thermal cycler (Applied Biosystems 9700).This 150 □l of solution was transferred to microplate just after the incubation, 50 □l of substrate solution (0.0040g Dopamine 1.0 ml distilled water) was added to the wells and the plate was incubated in dark at room temperature for 10 min and read in Microplate reader (Metertech Σ 960,USA) equipped with a 490 nM filter.

Optimum pH

Fifty microliter of hemolymph from normal and virosed larvae of *H. armigera* was taken and pre incubated for 30 min with 100□l of different buffers of pH 3.0 to 11.0. Citrate buffer 0.05 M was used for the range of pH 3.0 to 6.0, 0.1 M phosphate buffer was used for the range of pH 7.0 to 8.0.and sodium bicarbonate buffer 0.1 M was used for the range of pH 9.0 to 11.0. Due to autooxidation of substrate at an alkaline pH, the substrate blank was subtracted from the hemolymph absorbance.

PH Stability

The pH stability was studied by incubating the enzyme for 24 hr at room temperature in different buffers of pH 3.0 – 11.0.and assaying with 0.01M sodium phosphate buffer pH 7.0.

Study of *in vitro* inhibition of phenoloxidase

In this study all inhibitors were used in 2x concentration viz. (EDTA) 25mM, 2.5mM, 5mM, (EGTA) 25mM, 12.5mM, 5mM, (STI) 10mg, 1mg, 0.1mg, (PTU) 15mM, 7.5mM, 1.5mM, (PMSF) 25mM, 12.5mM, 1mM, (SDS) 1%, 0.5%, 0.05%, (DMSO) 10%, 1%, 0.1%, and Garlic extract, 10%, 5%, 1%. 25 □l of different concentrations of inhibitors were incubated at 30^0C at RT mixed with 25□l of hemolymph from normal and virosed larvae of *H. armigera* in each well of micro plate. Phosphate buffer (pH 7.0) 100□l of concentration 0.01M was added in each well. Afterward 50 □l of substrate solution was added to the plate and incubated in dark at room temperature for 10 min. and latter on it was read in microplate reader equipped with a 490 nM filter. Control was kept as 25□l of hemolymph plus 25□l distilled water instead of inhibitor mixture; reduction in the intensity of color developed due to addition of inhibitors was calculated.

Electrophoresis study

No denaturing polyacrylamide gel electrophoresis (PAGE) was done with Hoefer SE600 slab gel unit (Hoefer, San Francisco, CA) using buffer system, 10% running gel and 5% stacking gel was prepared according to manual provided with the Hoefer system. A crude hemolymph was loaded in the gel and electrophoresis was conducted (Bidochka *et al*., 1989) [13] at a constant current of 35mA (Hoefer PS-500 power unit) for 7-8 hr at 4ºC.

After electrophoresis, non-specific phenoloxidase band in the gels were revealed by incubating it in a solution of 40 mg of L-DOPA in 100 ml of 0.01 M Sodium phosphate buffer (pH 7.0) that was prepared and filtered just before used. The gel was incubated in the staining solution for 2 hr.

Results and Discussions

The PO activity in plasma fraction of both virosed and normal larvae of *H. armigera* was increased. However, in case of cellular fraction, it increased in 3rd instar larvae in both virosed and normal *H. armigera*, but in 5th instar the PO activity in cellular fraction decreased as the age of larvae increased, whereas the titer of PO in 5th instar larvae was more as compared to 3rd instar larvae (Table1). The higher activity of PO in NPV treated larvae was found to induct with onset of NPV infection.

Phenoloxidase is an enzyme that is involved in non-self-recognition in insects. In present investigation, the higher activity of PO in plasma fraction of hemolymph of 5th instar NPV treated *H. armigera* larvae than cell fraction seems to in responses to NPV infection and this was also demonstrated earlier in other insects, *S. litura, S. exigua, Melanoplus sanguinipes* infected with different microbes (Jyachandran *et al* 2000, Hung and Boucias 1996, Miranpuri *et al* 1997) [21, 21]. These higher levels of phenoloxidase in the plasma could lead to a more rapid and vigorous melanization-encapsulation reactions in responses to exposure to pathogens.

Characterization of PO is important in understanding this enzyme, which is needed for melanization and sclerotization. In addition, melanization may be important in insect defense responses. In present study crude hemolymph was used, for the characterization of phenoloxidase (PO) and hence the optimum values reported did not necessarily therefore, reflect the true parameter of certain PO, however, they represent the most favorable kinetic properties and assay condition for PO.

Normal larvae PO had a Km of 1.36mM where as virosed had Km of 1.21mM However, Vmax was 0.333 and 0.377 ΔA/mg protein/min, respectively, when L-tyrosine was used as a substrate. When L-Dopa was used as a substrate Km of 2.85mM and 1.25mM Km was found in normal and virosed larvae respectively where as Vmax found to be 0.667 and 0.832 ΔA/mg protein /min. respectively, Similar observation were made by Lockey and Ourth (1992) [29], found that Km for *H. virescens* larval hemolymph PO was 2.25mM and Vmax was 0.235 ΔA/mg protein /min, when L-Dopa was used as a Substrate, as was reported earlier by Morgan *et al*., (1990) reported 2.2mM Km for integument PO of *M. Sexta.* While, the Km of the hemolymph tyrosinase from *M. Sexta* was 4.5mM (Aso *et al*., 1984) [10].

Similar way Ashida and Dokhe (1980) reported that in *B. mori* hemolymph PO had Km of 1.7mM, 1.8 mM and 2.0mM beside Vmax was found to be 100,112, and 49, respectively at pH 6.5, 7.5 and 9.0 when L-Dopa was used as a substrate for hemolymph PO. Adamo (2004) [1] also found the Km of 2.5mM and Vmax of 5mM/min for hemolymph PO of *Gryllus texensi* at pH 7.4. Similar results were also noticed by Siva-Jothy *et al* (2001) [36] for phenoloxidase activity in damselflies.

During the present study when Methylcatechol was used as substrate Km was found to be 5.40mM and 1.66mM also Vmax was found to be 0.454 and 0.308 ΔA/mg protein /min for normal and virosed hemolymph from *H. armigera*. However, Pay (1978) reported Km of 1.49mM for immune *Galleria mellonella* larvae whereas normal larvae had a Km of 1.56mM same result regarding Km of virosed larvae were reported as for normal larvae where as during present study the km was found to be higher for normal larvae when Methylcatechol was used as a substrate. Nevertheless, Vmax reported by him was lower in case on immune as compared to normal one as against in our study Vmax found to be increasing in virosed larvae as compaired to normal. This might be due to more active enzyme from virosed larvae as against normal larvae. Barrett and Anderson (1981) [2] reported Km 0.22mM when Methylcatechol was used as a substrate in Blow fly *Calliphora vicica* for cuticle PO enzyme B, when L-Dopa was used as substrate Km found to be 1.67mM.

During current study when Dopamine was used as a substrate Km was observed as 6.23mM and 5.55mM for normal and virosd larvae whereas Vmax found to be 1.053 and 1.587 ΔA/mg protein /min respectively(Fig.1). Likewise Asno and Ashida (2001) reported that cuticular PO from *B. mori* had Km of 0.53mM for dopamine and for L-Dopa it was1.9mM, where Purified PO was used.

Dopamine showed maximum phenoloxidase activity in both normal (656.16 Unit/ mg protein/min) and virosed (902.65 Unit/ mg protein/min) *H. armigera* larvae. And ultimately maximum percent increase in PO activity (55.30%) in virosed as compared to normal hemolymph, was observed which was followed by L-Dopa, DL-Dopa, Methylcatechol and L-Tyrosine.

Hall *et al* (1995) [37] showed that for *M. sexta* PO, N-β-alanyldopamine, methyl catechol and dopamine as best substrate than Dopa. Chase *et al* (2000) [15] found that N-acetyl dopamine, 4-methylcatechal, dopamine were better substrates than Dopa while making assay of PO in *Sarcophaga bullata* Further they opined that unlike mammalian tyrosinase insect PO prefer Dopamine better than Dopa likewise, PO is typical O- diphenols oxidase possess monophenol monooxygenase activity. Also, Asano and Ashida (2001)[5] studied substrate specificity for cuticular PO for *B. mori* and found better substrate in descending order as dopamine, *N*-acetyldopamine, L-dopa, metylhydroquinone and hydroquinone.

An increase in enzyme activity was observed with increase in the temperature up to 40^0C and maximum PO activity was recorded at 40 ºC (1448.11 ΔA/mg P/min) in normal larvae. Similar trend of PO activity was also observed in virosed larvae, which showed 1640.49 Unit/ mg protein/min PO activity at 40 ºC. The PO activity both from normal and virosed hemolymph decreased after 50^0C and reached to minimum at 80^0C, 5.90 Unit/ mg protein/min in normal, and was not detected in virosed larvae suggesting possible denaturation and loss of active 3-D structure of PO enzyme at this temperature (Fig.2).

Same way Bidochka *et al* (1989) [13] found that PO activity of grasshoppers from subfamilies Melanoplinae and Oedipodinae was optimally active at temperature 37^0C. Lockey and Ourth (1992) [29] also reported that optimum temperature for *H. virescens* was 45^0C. Possible denaturation and loss of active 3-D structure of PO enzyme at temperature of above 40^0C could be the reason as activity was decreasing after 40^0C in virosed and normal larvae. Similar observations were made by Tsukamato *et al* (1986).

PO stability at different temporal temperatures indicated that the PO from normal larvae of *H. armigera* showed almost 100 % activity at 10 ºC, 20 ºC and 30ºC up to 80 min. thereafter the activity decreased. PO enzyme from virosed larvae also showed same pattern of enzyme stability as that of normal larvae. It was almost 100 % stable at 10,20 and 30 ºC up to 80 min interval. The enzyme activity was reduced afterwards (Fig 3 and Fig 4).

Phenoloxidase from normal and virosed larvae of *H.armigera* was stable up to 40^0C for about 40 minutes and its activity goes on decreasing when exposed to more than 40 minutes at 40^0C and even after 30 minutes at 50^0C. Above this temperature the protein was abruptly inactivated due to precipitation. Likewise Ashida (1971) [6] found that Pre-phenoloxidase from the hemolymph of *Bombyx mori* at 40^0C retained its original activity for 60 min, whereas at 50^0C, 20% of activity was diminished for 60 min and above 55^0C the protein was abruptly inactivated. Tsukamoto *et al* (1986), reported that latent phenoloxidase from the *Musca domestica* was stable at temperatures between 0 and 40^0C, whereas it was fairly unstable at temperature higher than 50^0C and lost 80 per cent of its activity at 60^0C when incubated for one hr at

this temperature. Chase *et al* (2000) [15] found that in *Sarcophaga bullata* PO enzyme rapidly lost its activity at room temperature (25^0C) within 15 min while raising temperature at 55^0C resulted in total loss of its activity within one min.

The optimum pH experiment showed that in normal *H. armigera*, the PO activity was 36.78 Unit/ mg protein/min at pH 3.0 and 784.84 Unit / mg protein/min at pH 11.0 The highest activity was found to be 902.20 Unit / mg protein/min at pH 9.0. The PO activity from virosed *H. armigera* was observed as 90.25 Unit/ mg protein/min at pH 3.0 and 800.97 Unit / mg protein/min at pH 11.0, the highest activity 922.75 Unit/ mg protein/min was found at pH 9.0 (Fig.5).

Optimum pH for PO activity was found to be 9.0 for normal and virosed *H. armigera* larvae. It was also observed from the data that PO was more active in alkaline pH as compared to acidic pH. Likewise Ashida (1971) [6] found that Pre-phenoloxidase from the hemolymph of *Bombyx mori* was stable between pH 5.8 and 9.0 whereas below pH 5.8 and above pH 9.0 inactivation of protein took place. Same way Tsukamoto *et al* (1986) [40] reported that phenoloxidase from the housefly *Musca domestica* was stable at pH 9.0.Lockey and Ourth (1992) [29] found that optimum pH for *H. virescens* larval hemolymph PO was 9.0.While Hung and Boucias (1996) [21] found that optimum pH for naïve and *Beauveria bassiana* infected *S. exigua* larvae in hemolymph PO to be 7.0. Whereas at pH 6.0 and pH 8.0 the decreased PO activity was recorded and at pH 4.0 no PO activity was detected. Same way Chase *et al* (2000) [15] also found that in *Sarcophaga bullata* the optimum pH for PO was 7.0.

The results on PO inhibitor specificity indicated that, amongst all Po inhibitors SDS at higher concentration of 1% was found to be the best than all other followed by EGTA >PTU> EDTA > PMSF> DMSO > Garlic extract. However the lower concentration of SDS i.e. 0.10 % and even 0.50 % did not inhibit PO and no inhibition due to STI was observed. In *H. armigera* detergent and chelating agent or serine protease inhibitors, which are believed to block the activation of Pro-PO to PO were found to inhibit PO activity.

Amongst all, SDS at higher concentration of 1% was found to be the best inhibitor than all other inhibitors studied, followed by EGTA >PTU> EDTA > PMSF> DMSO > Garlic extract. However the lower concentration of SDS i.e. 0.10 % and even 0.50 % did not inhibit PO.

Barrett and Anderson (1981) [2] reported similar types of result in Blow fly *Calliphora vicina*. They found that cuticle PO enzyme 'A' was inhibited by thiouria, phenylthiouria but less so by sodium azide and NaF. Same way Anderson *et al* (1989) [2] reported that PPO activation showed a bell-shaped dependence on EDTA or EGTA concentration with activation below and inhibition above 2mM.

Lockey and Ourth (1992) [29] found that the activity of PO for *H. virescens* larval hemolymph was not affected by calcium or EGTA, but both EDTA and SDS inhibited the activity. While Hung and Boucias (1996) [21] found that in naïve and *Beauveria bassiana* infected *S. exigua* larvae both potent serine inhibitor STI (1 mg/ml) and DFP (1,10 mM) suppress the PPO to PO activation, however during present investigation no inhibition was observed, due to STI which may be due to of differential PO activity as pointed out by Lockey and Ourth (1992) [29] who concluded that PO enzyme isolated from different species are not identical in their physical and chemical properties. Whereas, Nagai and Kawabata (2000) [32] reported that in horseshoe crab *Tachypleus tridentatus* PO activity was completely inhibited by typical inhibitor phenythiourea (12.5mM) and EDTA (25mM).

Asano and Ashida (2001) [5] found that cuticular PO in *B. mori* was completely inhibited by 1mM of phenylthiourea. Whereas Mantawy and Mahmoud (2002) [30] reported the effect of feeding of *Allium cepa* and *Allium sativum* on *Biompha alexandrina*, Phenoloxidase (PO) activity was significantly decreased after 2 and 7 days of feeding on garlic, whereas feeding on onion decreased the activity of the enzyme during all periods.

Electrophoretograph obtained after incubation of gel with L-Dopa revealed that two PO isozymes were present in hemolymph of normal and virosed *H armigera* larvae. In normal *H armigera* larvae, both isozymes (PON1 and PON2) migrated in close proximity with Rf values of 0.087 and 0.101, respectively. (Plate1).

Interestingly, hemolymph of virosed *H. armigera* also possesses two PO isozymes (POV1 and POV2). POV1 posses Rf value of approximately 0.096 similar to PON1 and PON2. Unlike POV1, POV2 posses very small Rf value 0.659 suggesting any activation because of HaNPV Infection.

In normal *H armigera* larvae both isozymes (PON1 and PON2) migrate in close proximity and poses higher molecular weight. Whereas hemolymph of virosed *H. armigera* posses two PO isozymes (POV1 and POV2). Amongst it POV1 posses Rf value approximately similar to PON1 and PON2 suggesting that it posses more or less same molecular weight. All high molecular weight PO isozymes (PON1, PON2 and POV1) can be of inactive Pro PO enzyme. Unlike POV1, POV2 posses very small Rf value and it's smaller molecular weight is suggestive of activated phenoloxidase obtained after the cleavage of prophenoloxidse.

Bidochka *et al* (1989) [13] reported similar types of results in PO activity in grasshoppers from subfamilies Melanoplinae and Oedipodinae. Chymotrypsin-activated hemolymph showed two bands of PO activity and one in non-activated hemolymph .The additional band due to chymotrypsin-activated migrated further in the native gel, suggested that it posses low molecular weight as compared to inactive (PPO) enzyme.

Table 1: Phenoloxidase activity in cellular and plasma fraction of Normal and Virosed 3rd & 5th instar larvae of *H. armigera*

Post Infection Period	Normal 3rd instar (Unit/mg protein/min)		Virosed 3rd instar (Unit/mg protein/min)		Normal 5 th instar (Unit/mg protein/min		Virosed 5 th instar (Unit/mg protein/min	
	Cellular	Plasma	Cellular	Plasma	Cellular	Plasma	Cellular	Plasma
0hr.	322.99±3.3	N.D*	325.41±4.9	N.D	427.04±2.7	229.18±3.8	645.71 ±4.6	234.32 ±5.7
24hr	346.02±2.6	N.D	680.17±4.9	21.62±2.14	486.18±2.5	277.93±3.9	621.43 ±4.9	319.74 ±5.3
48hr	361.49±4.7	N.D	807.37±3.0	168.6±4.07	506.7 ±3.2	391.33±43	889.89 ±4.6	732.77 ±6.1
72hr	411.32±3.5	152.63±3.3	902.24±3.7	272.2±3.67	431.49±2.0	392.04±4.1	508.99 ±4.1	968.34 ±6.1

***** N.D- Not Detected

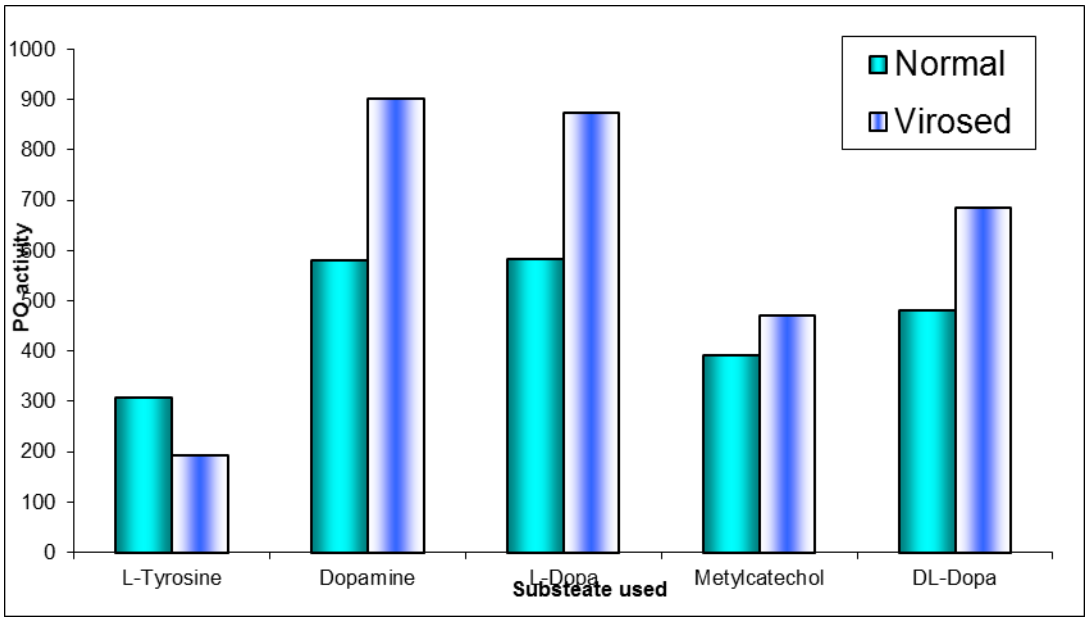

Fig. 1: Substrate specificity of phenoloxidase (PO) from normal and virosed V instar larvae of *H, armigera*

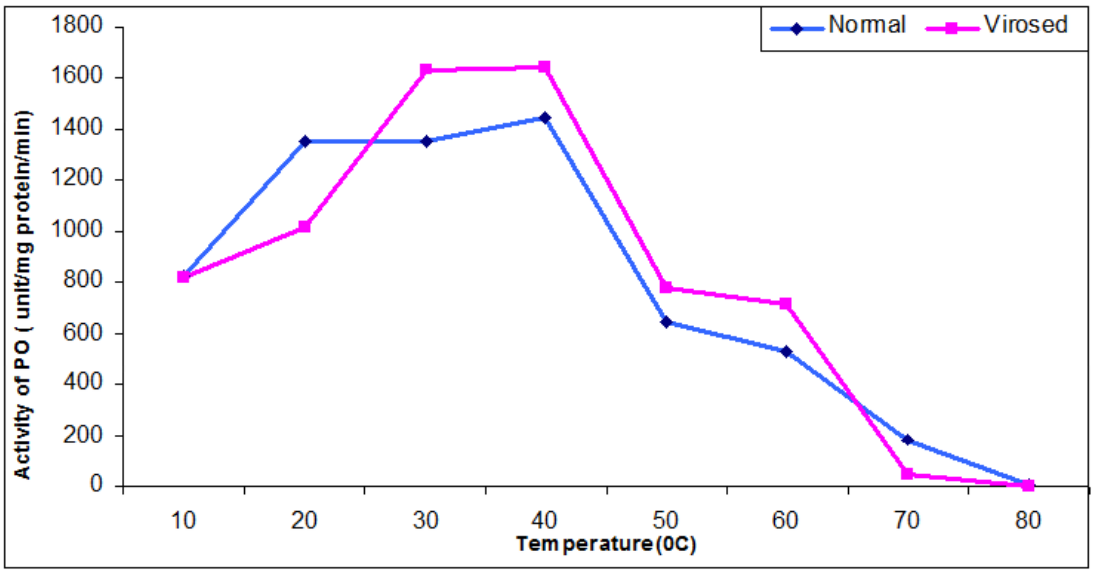

Fig. 2: Effect of temperature on hemolymph phenoloxidase activity from normal and virosed V instar larvae of H.armigera

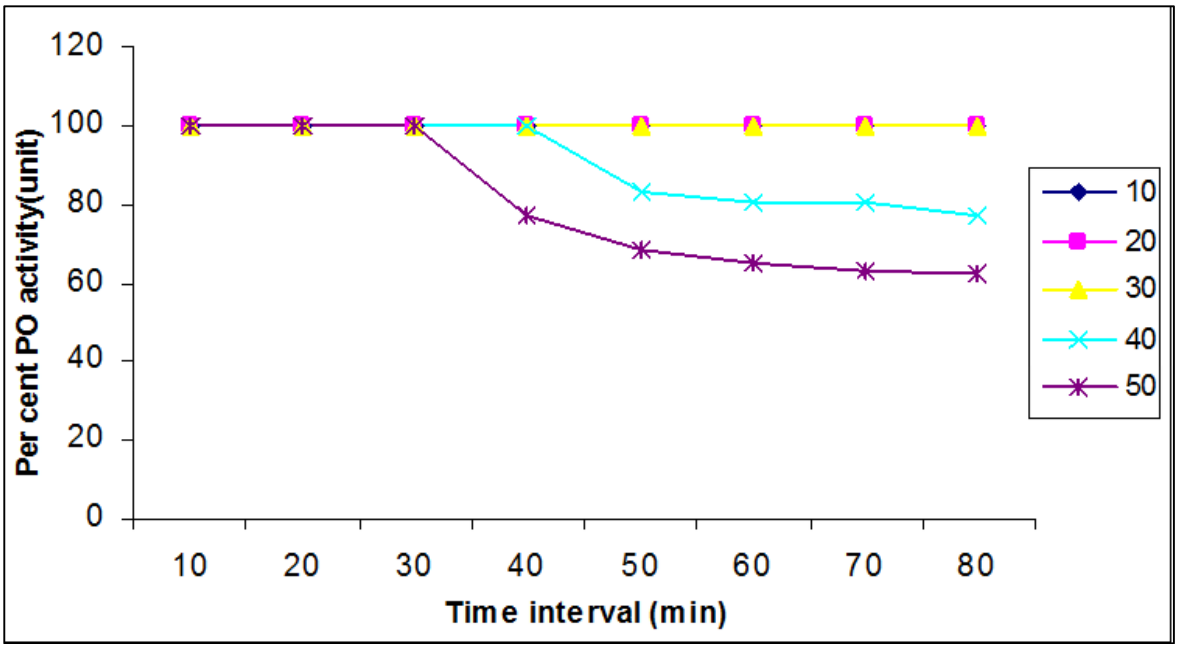

Fig. 3: Thermal stability of the phenoloxidase enzyme from normal V instar larvae of H. armigera

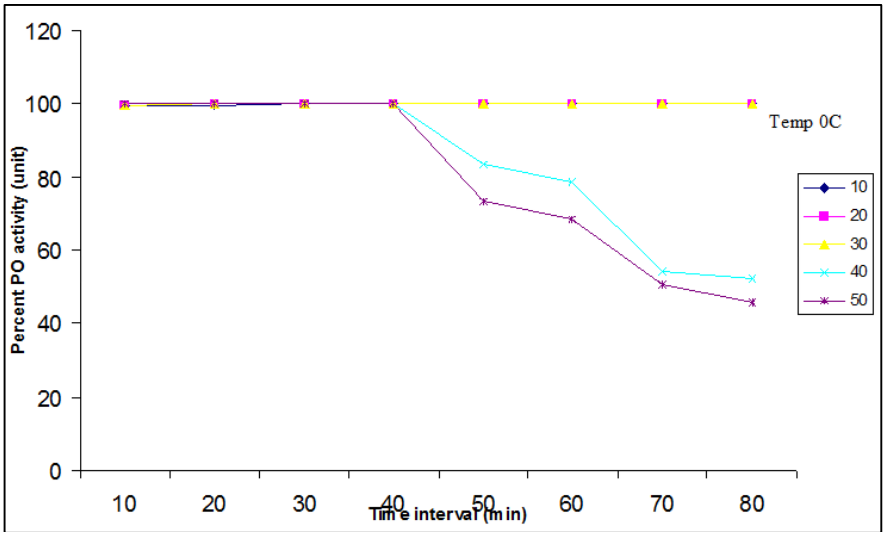

Fig. 4: Thermal stability of the phenoloxidase enzyme from virosed V instar larvae of *H. armigera*

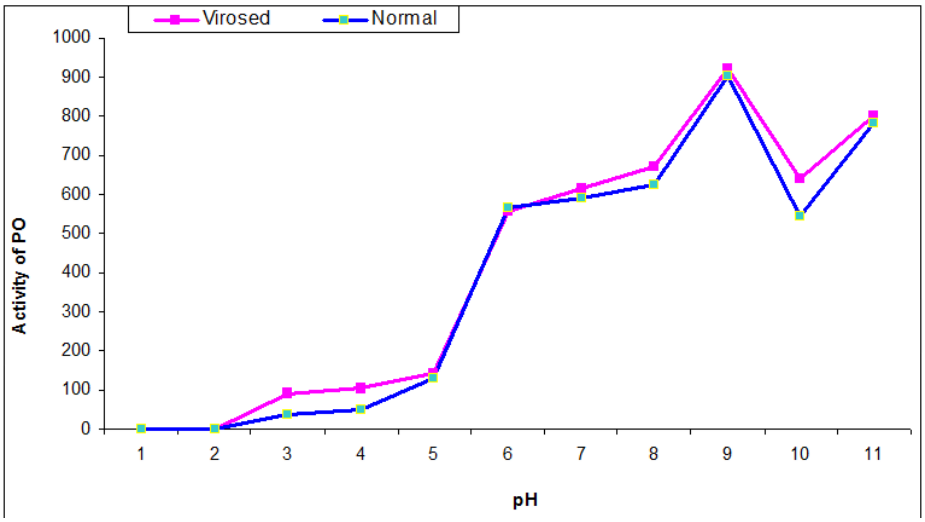

Fig. 5: Effect of pH on PO activity from normal and virosed V instar *H. armigera* Larvae

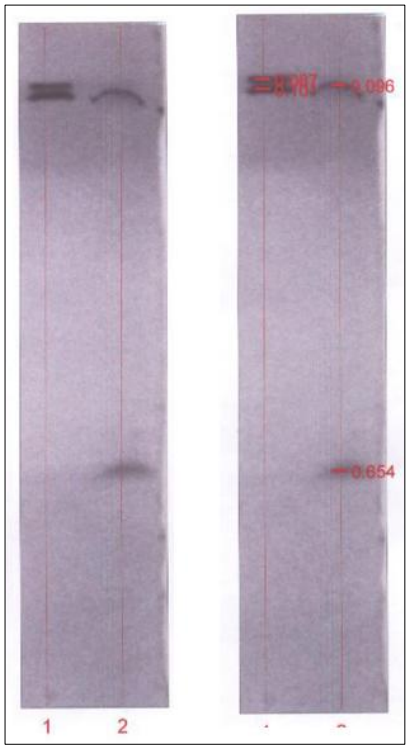

Plate 1. : Electrophoretograph obtained after incubation of gel with L-Dopa reveled that two PO isozymes were present in hemolymph of normal and virosed *H armigera* larvae.

Conclusion

For the first time the characterizations PO of *H. armigera* from hemolymph was carried out, these studies will go as foundation for further studies to understand the enzymatic and molecular basis of the PO enzyme. Moreover it provides a better understanding of physical and chemical properties of this enzyme, which is needed for important reaction of cell communication, sclerotization and melanization, which is concerned with immunity in insect.

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