STUDIES ON PHENOLOXIDASE ACTIVITY AND MELANIZATION IN *Helicoverpa armigera* (Hubner) EXPOSED TO HaNPV

THESIS

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DECLARATION OF STUDENT

I hereby declare that the experimental work and its interpretation of thesis entitled **"STUDIES ON PHENOLOXIDASE ACTIVITY AND MELANIZATION IN *Helicoverpa armigera* (Hubner) EXPOSED TO HaNPV"** or part thereof has not been submitted to any other university for award of any diploma or degree. The data of this experimental work have not been derived from any thesis or publication of any university or scientific organization. The sources of material used and all assistance received during the course of investigation have been duly acknowledged.

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CERTIFICATE

This is to certify that the thesis entitled "**STUDIES ON PHENOLOXIDASE ACTIVITY AND MELANIZATION IN *Helicoverpa armigera* (Hubner) EXPOSED TO HaNPV**", submitted in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Agriculture to Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, is a record of bonafide research work carried out by **Mr. Ajay Krishnakumar Sadawarte** under my guidance and supervision. The subject of the thesis has been approved by the student's advisory committee.

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TABLE OF CONTENTS

CHAPTER	PARTICULAR	PAGE
I	INTRODUCTION	1-4
II	REVIEW OF LITERATURE	5-30
III	MATERIAL AND METHODS	31-45
IV	EXPERIMENTAL FINDINGS	46-75
V	DISCUSSION	76-93
VI	SUMMARY	94-99
	LITERATURE CITED	100-110
	APPENDICES	
	VITA	
	THESIS ABSTRACT	

LIST OF TABLES

TABLE	TITLE	PAGE
1	Semi-synthetic diet for rearing of *H. armigera* larvae	37
2	Adult diet for *H. armigera* moth	37
3	Composition of vitamin stock solution	38
4	LD_{50} of NPV to *H. armigera* larvae	47
5	Phenoloxidase activity in cellular and plasma fraction of normal and virosed 3rd instar larvae of *H.armigera*	49
6	Phenoloxidase activity in cellular and plasma fraction of normal and virosed 5th instar larvae of *H.armigera*	51
7	Kinetics of haemolymph phenoloxidase from virosed and normal V instar larvae of *H. armigera* (substrate L-tyrosine)	53
8	Kinetics of haemolymph phenoloxidase from virosed and normal V instar larvae of *H.armigera* (substrate L-dopa)	55
9	Kinetics of haemolymph phenoloxidase from virosed and normal V instar larvae of *H.armigera* (substrate Methylcatechol)	57
10	Kinetics of phenoloxidase from virosed and normal haemolymph of V instar larvae of *H. armigera* (substrate Dopamine)	59
11	Kinetics of phenoloxidase from virosed and normal haemolymph of V instar larvae of *H. armigera* (substrate DL-Dopa)	61
12	Michaelis constant (Km) and maximum velocity (Vmax) of phenoloxidase from normal and virosed V instar larvae of *H. armigera.*	63
13	Substrate specificity of phenoloxidase (PO) from normal and virosed V instar larvae of *H, armigera*	64
14	Effect of temperature on haemolymph phenoloxidase activity from normal and virosed V instar larvae of *H.armigera*	65
15	Thermal stability of the phenoloxidase from normal V instar larvae of *H.armigera*	67
16	Thermal stability of the phenoloxidase from virosed V instar larvae of *H.armigera*	67
17	Effect of pH on PO activity from normal and virosed V instar larvae *H.armigera*	69
18	Phenoloxidase and HaNPV toxicity from different chromotonal virosed V instar *H. armigera* larvae	71
19	Effect of different inhibitor on phenoloxidase activity	72
20	Total haemocyte count mm^3 of haemolymph in normal and virosed larvae of *H. armigera*	74

LIST OF FIGURES

FIGURE	TITLE	PAGE
1	Probit Line of different instar of *H.armigera* larvae	48
2	Phenoloxidase activity in cellular and plasma fraction of normal and virosed 3rd instar larvae of *H.armigera*	50
3	Phenoloxidase activity in cellular and plasma fraction of normal and vrosed 5th instar larvae of *H.armigera*	52
4	Lineweaver-Burk plot for PO activity of normal V instar larvae of *H. armigera* by using L-tyrosine as substrate	54
5	Lineweaver-Burk plot for PO activity of virosed V instar larvae of *H. armigera* by using L-tyrosine as substrate	54
6	Lineweaver-Burk plot for PO activity of normal V instar larvae of *H. armigera* by using L-Dopa as substrate	56
7	Lineweaver-Burk plot for PO activity of virosed V instar larvae of *H. armigera* by using L-Dopa as substrate	56
8	Lineweaver-Burk plot for PO activity of normal V instar larvae of *H. armigera* by using Methylcatechol as substrate	58
9	Lineweaver-Burk plot for PO activity of virosed V instar larvae of *H. armigera* by using Methylcatechol as substrate	58
10	Lineweaver-Burk plot for PO activity of normal V instar larvae of *H. armigera* by using Dopamine as substrate	60
11	Lineweaver-Burk plot for PO activity of virosed V instar larvae of *H. armigera* by using Dopamine as substrate	60
12	Lineweaver-Burk plot for PO activity of normal V instar larvae of *H. armigera* by using DL-Dopa as substrate	62
13	Lineweaver-Burk plot for PO activity of virosed V instar larvae of *H. armigera* by using DL-Dopa as substrate	62
14	Substrate specificity of phenoloxidase (PO) from normal and virosed V instar larvae of *H. armigera*	64
15	Effect of temperature on haemolymph phenoloxidase activity from normal and virosed V instar larvae of *H.armigera*	66
16	Thermal stability of the phenoloxidase enzyme from normal V instar larvae of *H.armigera*	68
17	Thermal stability of the phenoloxidase enzyme from virosed V instar larvae of *H.armigera*	68
18	Effect of pH on PO activity from normal and virosed V instar *H.armigera* larvae	70
19	Total haemocyte count mm^3 of haemolymph in normal and virosed larvae of *H. armigera*	75

LIST OF PHOTOGRAPHIC PLATES

PLATES	TITLE	AFTER PAGE
1	Rearing and Bioassay of *H. armigera*	36
2	Microplate Assay of Hemolymph Protein and Phenoloxidase	40
3	Haemolymph PO isozymes pattern from Normal and Virosed larvae of *H. armigera*	73
4	Haemocytes from *H.armigera*	75
5	Haemocytes from *H.armigera*	75

ABBREVIATIONS AND ACRONYMS

a.i.	- Active Ingredients
SE (±)	- Standard error
et al.	- et alia (and others)
hr	- Hour
i. e.	- that is
fig.	- Figure
PO	- Phenoloxidase
proPO	- prophenoloxidase
l	- Liter
LD_{50}	- Lethal dose for killing 50 per cent population
LD_{90}	- Lethal dose for killing 90 per cent population
ldp	- Log dose probit
μg	- Micro gram
μl	- Micro liter
μmol	- Micro molar
mg	- milligram(s)
ng	- Nano gram
N. S.	- Non-significant
PIBs	- Polyhedral inclusion bodies
POBs	- Polyhedral occlusion bodies
Sig.	- Significant
Viz.	- Videlicet (namely)
HaNPV	- *Helicoverpa armigera* nuclear polyhedrosis virus
^{o}C	- Degree centigrade
%	- Per cent
LE	- Larval equivalent
NPV	- Nuclear polyhedrosis virus
Rpm	- Revolution per minutes
Vmax	- Maximum velocity
Km	- Michaelis constant
OD	- Optical density
mM	- milimolar
nM	- nano molar
μM	- micromolar
min	- minutes
OB	- occlusion bodies
EC	- Emulsifiable concentrate
AC	- anticoagulant
ΔA/mg/min	- Differences in absorbance's per milligram(s) per minutes
PCR	- Polymerase chain reaction
Cm.	- Centimeter
THC	- Total haemocytes count

CHAPTER I

INTRODUCTION

Insects have demonstrated considerable ability to develop resistance to conventional insecticides and more than 654 species of insects and mites have already developed resistance to one and more chemicals. But can an insect species, susceptible to a pathogen develop resistance to more microorganisms? Host specificity observed with many insect pathogens demonstrated that insect species are naturally resistant to these microorganisms (Narayanan, 2004). 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.

Insect immune system is associated with cellular and humoral components. Insect lack specific immunoglobulins, instead they rely upon binding proteins present in the plasma or haemocytes to recognize non- self material (Ratcliffe *et al.*, 1985). This recognition leads to the activation of the immune system, involving cellular events such as phagocytosis and nodulation, and humoral events such as activation of prophenoloxidase cascade in the haemolymph that produces effectors molecules such as cytotoxic quinones which leads to melanization (Ashida and Brey, 1998). Besides haemocytes, the measurement of phenoloxidase and lysozyme like enzyme activity in the haemolymph has often been used to estimate disease resistance (Rantala *et al.*, 2002).

Phenoloxidase (PO), also known as tyrosinase, is a bifunctional enzyme possessing both monophenol and monooxygenase (E.C.1.14.18.1 tyrosine, dihydroxyphenyalanine, oxygen, oxidoreductase) and *o*-diphenoloxidase activity (E.C.1.10.3.1.o-diphenol, oxygen, oxidoreductase), and also uniquely associated with three different physiologically important biochemical processes in insect and other arthropods. These are sclerotization of insect cuticle (Anderson et al., 1996; Sugumaran, 1998), encapsulation and melanization of foreign organisms observed as a defense reaction (Ashida and Brey,1995; Gillespie *et al.*, 1997; Soderhall *et al.*,1990) and wound healing (Lai-Fook,1966; Sugumaran,1996).

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 and therefore necessary for insect management.

The deletion of insecticides from our arsenal due to environmental concerns or development of resistance in target insects necessitates continuing discovery of new pest management strategies to maintain the sustainability in agriculture. This changing scenario in agriculture has lead to an urgent need to develop ecologically sound, economically viable, socially acceptable and non-conventional but considerably effective pest management strategies (Singh *et al.*, 1999).

Helicoverpa armigera (Hubner) Hardwick is the most dreaded pest of almost all the cultivated crops and has attained global importance as alarming pest. It has been recorded to feed on 181 cultivated and non–cultivated plant species (Manjunath *et al.*, 1989). Several insecticides have been screened and advised against *H.armigera* (Sachan, 1992 and Pawar *et al.*, 1999) from time to time. However, exclusive dependence on chemical insecticides and their injudicious and unsystematic use led to the problems like development of manifold resistance in *H. armigera* to almost all the available insecticides (Armes *et al.*, 1996 and McCaffery, 1999), pest resurgence and destruction of beneficial fauna, pesticide residue and environmental pollution. Development of such methods has predicted the necessity for more research on biological control with hopefulness that the reliance on chemical pesticides will be reduced. Amongst bio-agents, insect pathogens have been comprehensively studied and extensively used to partly substitute the use of insecticides.

The insect viruses from 'Baculoviridae' form a novel group of microbial agents. They produce fatal disease in larvae of a number of insect species, particularly in Lepidoptera and Hymenoptera. The infection cycle of baculoviruses is mediated by two phenotypically different virus particles, occlusion derived virions (ODV), which establish initial infection through the midgut and budded virions (BV), that spread infection within the host. Occlusion derived virions are contained within the polyhedral inclusion bodies (PIBs) called polyhedra. The polyhedra infect larvae of susceptible species following ingestion. The alkaline juices within the midgut lumen dissolve the PIBs and liberate the ODV, which infect mature and differentiating midgut columnar epithelial cell. Thereafter, infected midgut cells produce BV,

which transmit infection to other larval tissues. BV infection of larval tissues results in to the production of more BV and hundreds of millions of polyhedra. These polyhedra are released into the environment upon liquification of host (Monobrullah and Nagata, 2000).

In India, field trials of nucleopolyhedrosisvirus (NPV) were carried out against *H.armigera* on various crops with varying results (Rabindra *et al.*, 1994; Panchabhavi *et al.*, 1995 and Datkhile, 2000).Despite the demonstration of their potential, the baculoviral formulations have not found widespread use in pest management which can be attributed to I) *Per os* mode of action requiring extensive coverage of the crop foliage II) Poor persistence in the field as solar radiation causes degradation HaNPV thereby limiting its virulence and persistence in nature and requiring more frequent sprays III) Resistance in older larvae as larvae generally show reduction in susceptibility to baculovirus infection with increase in age providing a narrow window for timing the sprays (Narayanan, 1979, Jayachandran and Chaudhari, 1996, Datkhile, 2000, Kalia and Chaudhari, 2001 and Rabindra 2003).

Decrease in susceptibility on increase in age was largely linked to increasing body weight (Ignoffo, 1966 and Evans, 1981,). Secondly, the infectivity was related with the phenotype of virus particle i.e. the occlusion derived virions (ODV) and the budded virions (BV). The BV was highly infectious as compaired to ODV, when injected into the haemocoele whereas ODV was more infectious than BV when administered orally to the same insect. Thus, the two forms reflected different functional requirement both in survival and cell attachment in the alkaline environment of the gut and the slightly acidic condition in the haemolymph. Therefore, the gut appeared to act as a barrier to infection in late stage of larval development (Monobrullah and Nagata, 2000). Likewise, the physiological changes associated with pupation may not allow infestation at this stage and apparent total resistance of older larvae due to the fact that pupation takes place before the virus could exert its influence on larvae (Gitanjali Jayachandran and Chaudhari, 1996).

Besides, there are various pre-gut and post-gut factors, which may be the possible candidates for conferring resistance to virus infection in older larvae (Prasad and Ramakrishinan, 1993). Further phenoloxidase (PO) system appeared to have an important role in supplementing the gut barrier by retarding secondary NPV infection (by the viron that manage to gain entry) in the haemolymph (Gitanjali Jayachandran *et al.*, 2000).

Inhibition of PO *in vivo* results in impairment of encapsulation responses (Brewer and Vinson, 1971), by blocking quinone formation by phenylthiourea an insect's parasite resistance can be suppressed (Pay, 1974). The haemocytes encapsulation responses in the clearance of viral pathogen suggested a possible strategy where by baculoviruses can be genetically manipulated to become a more efficacious biopesticides (Washburn *et al.*, 1996). So better understanding of the current knowledge of insect molecular genetics and molecular basis of the insect biochemical and cellular and humoral defense mechanism pave the way for the proper management of the pests, through the use of various biocontrol agents like parasitoids and pathogens after knowing how can they bypass the insect defense mechanism. Studies on insecticide resistance in insects and the mechanisms involved in it are available, however work on insect immunity or insect defense system is very scarce and its exploitation for the ultimate goal of insect pest management seems to be restricted.

Therefore, with these ideas in view, present studies were intended to discover I) LC_{50} values of HaNPV for different instars of *H.armigera* II) Role of PO in age related resistance, III) Characterization of PO, IV) Phenoloxidase activity and HaNPV toxicity in different chromotonal *H. armigera* larvae, V) Inhibition studies of PO, VI) Effect of different phenoloxidase inhibitors in combination with HaNPV on *H.armigera* larvae and VII) Effect of virus infection on Total haemocyte count. The findings of this study may give a spark in upcoming research to increasing the efficacy of HaNPV by bypassing the defense mechanism and making its host more vulnerable, in future.

CHAPTER II

REVIEW OF LITERATURE

Helicoverpa armigera [Hubner] Hardwick is dreaded polyphagus pest damaging all most every economic crop in India and inflicting a huge economic loss. In recent past its management has become increasingly difficult due to development of resistance to all major group of insecticides, due to which, thrust is given on biocontrol agent like HaNPV. But problem like age dependent resistance is one of the reason to reduce its efficacy. So to evaluate the role of phenoloxidase, activity and melanization in *Helicoverpa armigera,* when exposed to HaNPV, its characterization and inhibition were studied. The relevant available literature on this topic from India and abroad is summarized below

2.1. Age Dependent Toxicity of HaNPV to *Helicoverpa armigera*

Tanada (1956) studied some factors affecting the susceptibility of the armyworm *Pseudaletia unipuncta* to virus infection. It was found that, the age of larvae affects the susceptibility to virus infection. The average mortality caused by NPV in first, second, third, fourth, fifth and sixth larval instars were 85.8,78.1,31.5,25.8,16.1 and 3.4 per cent, respectively. The fifth and sixth instar were highly resistance.

Stairs (1965) observed the relative susceptibility of larval instars of forest tent caterpillar *Malacosoma disstria* (Hub) to NPV. It was found that susceptibility decreased markedly as larvae grow elder. The first instar larvae were 1000 times more susceptible than third instar larvae and about 68,000 times more susceptible than fourth instar.

Ignoffo (1966) studied the effect of age on the mortality of *Heliothis zea* (Boddie) and *H.virescens*. He found that all the stages of *Heliothis* exposed to the constant virus dose were susceptible to the NPV. As the larvae mature there is decrease in susceptibility as measured as either decrease in mortality or decrease in time for initial mortality. All the 1 to 6 day old bollworm and 99 % of 1 to 6 day old bud worms died when exposed to a constant dose of 2598 PIB/mm^2 or 1247 PIB/mm^2 respectively.

Pawar (1970) Worked on structural, developmental and physico-chemical properties of the nuclear polyhedrosis virus of *Spodoptera litura* (Fabricius) and associated changes in

the haemolymph of the host and found that the LC_{50} values were 0.333×10^6 and 13.33×10^6 PIB/ml for newly hatched and 5-day-old larvae of *S. litura* on 10 days of observation respectively. Merely on the basis of LC_{50} values, the calculated relative susceptibility to newly hatched larvae was, 40 times more than the 5 day old larvae.

Rabindra and Subramaniam (1974) conducted studies on Nuclear Polyhedrosis virus of *Heliothis armigera* (Hub) susceptibility and gross pathology. Incubation period increased with age whereas the susceptibility decreased as the larvae grew older. The mortality was found to be decreased as the age increased which was 100-96 % in 1st to 3rd instar whereas 80 % in 4th instar and just 20 % in 6th instar larvae of *H. armigera.*

Komolpith and Ramakrishanan (1975) performed bioassay of nuclear polyhedrosis virus against larval stage of *Spodoptera litura* (Fabricius) and the effect of protectants against ultraviolet light. The LC_{50} values for 4, 5 and 7-day-old larvae of *S.litura* were, 4.677, 13.70, and 37.240×10^6 polyhedral inclusion bodies (PIB)/ml respectively. The adjusted relative susceptibility, on the basis of relative amount of food consumed by the various age groups of larvae was found to be 1, 10.8,and 88.5 for 7, 5, and 4day old larvae. The relative susceptibility was calculated taking in to consideration the amount of contaminated food consumed by the different instar.

Whitlock (1977) tested the effect of larval maturation on mortality induced by NPV on *H. armigera,* including all the larval stages and found an inverse relationship between mortality and larval age (weight). This decrease in susceptibility can be measured either as decrease in mortality or to certain extent as an increase in incubation period. In this study larvae from 1 to 13 day age groups were exposed to constant dose of 2220 PIB/ml of the medium surface, the resultant average mortality percentage lowered down from 100 to 0 percent.

Evans (1981) studied the responses of all larval stages of *Mamestra brassicae* to doses of nuclear polyhedrosis virus (NPV). There was a 34,000- fold difference in LD_{50} values in the first (7 polyhedra) to the fifth instar (238,370 polyhedra) larvae. Although mid-fifth and sixth-instar larvae were fed doses up to 5×10^7 polyhedra, very little mortality was recorded and thus fifth and sixth instar larvae were virtually resistant.

Bucher and Turnock (1983) studied the relationship between the doses of a multi embedded nuclear polyhedrosis virus and mortality for each instar of the Bertha armyworm.

They made probit analysis, following the ingestion of polyhedral inclusion bodies (PIBs) placed on the surface of an artificial diet. Susceptibility to infection decreased, as larvae aged. The median exposure dose (LE_{50}) increased from 18 PIBs/larvae for the first larval instar to 21 x 10^6 PIBs/larvae for 6th instar. Whereas for 2nd, 3rd, 4th and 5th instar larvae the LC_{50} doses were 235, 444, 519 and 14130 PIBs/larvae respectively.

Flattery (1983) determined NPV activity in third and fourth instar larvae of *H. armigera*. It was found that, the third instar larvae of *H. armigera* were 100 times more susceptible than the fourth instar. The minimum time required to get the mortality was 4 days.

The relative susceptibility of *H.armigera* to different dosage of NPV and its biochemical effect with various larval instars was studied. It was found that the second instar larvae were more susceptible than third and fourth instar at lower, dosages of virus (Mowad et al.,1984).

Khaire (1985) made bioassay of NPV against newly hatched, 2,4,and 6 days old larvae of *H. armigera* and observed that, the LC_{50} values for these age groups were 0.21,1.67,2.34 and 3.88 x 10^7 PIBs/ml, respectively. Further, it was reported that mortality of the larvae increased with increase in the dosage.

Teakle *et al.*, (1985) studied the susceptibility of *H. armigera* larvae of different ages to a commercial nuclear polyhedrosis virus (NPV) 'Elcar', by conducting bioassay. The median lethal dosage (LD_{50}) increased 150-fold during the first week of larval life. For first instar larvae the dose was 5.0 x 10,where as for second, third, fourth and for fifth instar larvae it was 1.4 x 10^2, 2.3 x 10^3, 5.1 x 10^3 and 3.4 x 10^6 polyhedra respectively, indicating a progressive increase in the virus dose required to infect larval age subsequently. Resistance to infection developed more rapidly with increasing larval age.

Teakle *et al.*, (1986) studied the age-related susceptibility of *Heliothis punctigera* to a commercial formulation of nuclear polyhedrosis virus. The medial lethal dosages (LD_{50s}) showed a general increase with advancement in larval instar newly hatched larva showed a 2-fold increase in LD_{50} in first 6 hr, and there was 160-fold increase from newly hatched to newly molted fourth instar larvae. In general instar wise LD_{50} values obtained were 7.3 x 10, 1.2 x 10^2, 1.1 x 10^3, 6.0 x 10^3 and 2.1 x 10^6 polyhedra for first, second, third, fourth and fifth instar respectively.

Salama *et al.*, (1986) studied the effect of NPV on various stages of the *H. armigera* in the laboratory. It was observed that, the first instar larvae showed more susceptibility to virus than the second instar. However, mortality was increased with increase in viral concentration.

Pathogenicity of NPV against *Spodoptera mauritia* was assayed by Nair and Jacob (1987) on 2nd, 3rd, 4th 5th and 6th instar larvae. It was found that, susceptibility to virus infection decreased with larval age. LC50 values ranged from 3558 POBs/ml for 2nd instar to 80,87,000 POBs/ml for 6th instar larvae.

Abbas and El-Dakroury (1988) investigated the efficacy of *H. armigera* NPV, the microbial insecticide 'Elcar' on different larval stages. Mortality occurred within 4-10 days after infection and it enhanced with an increase of dosage. At concentration of 2.2×10^8 PIBs/ml of NPV, the mortality reached 100 per cent in second and third instars larvae and 93 per cent in fourth instar larvae. When 'Elcar' was used at 1×10^5, 1×10^6 and 1×10^7 PIBs/ml, mortality for second instar larvae was 76, percent for the first and 100 per cent for the latter two dosage, respectively.

The efficiency of NPV for control of some insect pests in the Konkan region of Maharashtra state was studied by (Mehandale *et al.*, 1992). NPV bioassay against larvae of *Spodoptera litura* revealed that LC_{50} values for the 5th day old larvae was 6.224×10^6 PIBs/ml. It was observed that, the larvae in the younger age were more susceptible than older age. Larval mortality was 100 per cent at 18.75×10^6 PIBs/ml viral dose up to 7 days old larvae. The larval mortality decreased gradually and it was noted that the virus concentration of 18.75×10^6 PIBs/ml could not produce any mortality in 15 day old larvae.

Jayachandran and Chaudhari (1996) studied the effect of age-related response of *Spodoptera litura* Fab. larvae on their susceptibility to nuclear polyhedrosis virus. Bioassays conducted to determine the Pathogenicity of NPV on different larval age group of *S. litura* revealed that the LC_{50} values were found to increase with host age. The finding shows that LC_{50} values for 3,5,8,and 10 day old larvae were 7.57×10^4, 3.33×10^5, 2.23×10^7 and 5.08×10^8 PIB/ml respectively. As the host age increased, the susceptibility to the virus was found to decrease.

Dere (1997) conducted bioassay of NPV to assess the LD_{50} value for early third instar larvae of *H. armigera*. The LD_{50} value for early third instar larvae was 1×10^9 PIBs/ml.

Kencharaddi and Jayaramaiah (1997) carried out bioassay of nuclear polyhedrosis virus with first, third, and fifth instar larvae of *Adisura atkinsoni* and *H. armigera*. The LD_{50s} of nuclear polyhedrosis virus for the respective larval instars larvae of *A.atkinsoni* were 4.93 x 10^4, 8.28 x 10^4 and 2.17 x10^5 PIBs/ml, while for larvae of *H. armigera* these values were 2.9 x 10^4, 5.33 x 10^4 and 2.7 x 10^5 PIBs/ml, respectively. The LT_{50s} for these species varied from 5-10 and 4-12 days, respectively, depending on dosage for larval instars.

Landge (1998) on the basis of his laboratory studies reported an inverse relationship between mortality and larval instars of *H. armigera* to a constant dose of 1x 10^5 POBs/larvae. The LT_{50} values obtained for second, third, fourth, and fifth instar larvae were 116.86, 147.53, 191.77 and 212.25 h, respectively. Thus it clearly indicated that LT_{50} increased with increase in larval age.

Datkhile (2000) performed studies on the virulence of *Helicoverpa armigera* nuclear polyhedrosis virus. Bioassay for second, third, fourth and fifth instar larvae exhibited increased mortality in each instar with an increased NPV dose (POB/larvae). It was found that the LD_{50s} of NPV for second, third, fourth and fifth instar were observed to be 0.30 x 10^3, 1.42 x10^3, 6.13 x 10^3 and 47.6 x 10^3 POBs/larva respectively. The susceptibility of *H. armigera* second instar larvae was found to be 4.7 times more than thirds, 20.25 times than fourth and 157.1 times than fifth instar larvae.

Monobrullah and Nagata (2000) observed the developmental resistance in orally inoculated mature larvae of *Spodoptera litura* Fabricius with its nuclear polyhedrosis virus (NPV). The LD_{50}values of the second, third, fourth and fifth instar larvae were 224, 806, 20692 and 519381 PIBs/larvae respectively, indicating a steady increase with larval age. Thus, the second instar larvae were about 3.5, 92 and 2314-fold more susceptible than the third, fourth and fifth instar larvae respectively. No mortality was recorded through oral inoculation of the sixth instar larvae at any of these two PIB count tested (4.8 x 10^6 PIB/larva and 4.8 x 10^7 PIB/larva).

Kalia and Chaudhari (2001) studied the effect of larval age on mortality of *H.armigera* larvae exposed to nucleopolyhedrovirus. The susceptibility of American bollworm, *H. armigera* larvae of different ages to NPV was determined by bioassay. The LC_{50} value was found to increase with host age. The result showed that LC_{50} values of 1,3,5 and 8 day old larvae were 16.96, 4.54 x 10^2, 5.67 x 10^3 and 2.06 x 10^5 $OBml^{-1}$respectively.

One day old larvae, were 26.76, 334 and 121,00 times more susceptible than 3,5, and 8 day old larvae respectively, However, there was steady increase in LC_{50} values as the age increased from one to eight days. Thus the susceptibility was found to be decrease as age of larvae increased.

Vinodkumary and Singh (2002) evaluated the effectiveness of Biovirus (SlNPV) against the different age group of *S. litura* larvae. The susceptibility of the laryae was negatively correlated with larval period of development. One-day-old larvae were 2.7, 277.0 and 61951.2 time more susceptible than three, five and eight day old larvae, respectively on the basis of LC_{50} values.

2.2 Phenoloxidase and its characterization

2.2.1 Role of phenoloxidase and melanization

The ubiquitous presence of both infectious organisms and parasites in ecosystem occupied by insects exert a strong selection pressure for insects. But resistance to this infection is achieved with defensive immune system (Dunn, 1986).

As far as historical background of the insect immunity is concerned, the studies on insect immunity has started way back in 1920's, however, in 1950's it was discovered that certain immunity could be induced in some insects. Later on in 1980's Isolation and purification of induced antibacterial factor has been done (www.upsala.edu.). In short, immune system plays an important role in insect life as follows.

Defensive responses of insects

Immunity in insect differ from mammals in that insect do not produce lymphocytes. The defensive mechanism of insects contains both passive structural barriers and a cascade of active response to organism. The first line of defense includes the prevention of infection via passive structural barrier (Whitecomb *et al.*, 1974). These are Exoskeleton and Peritrophic membrane. The second line of defense includes cascade of active response i.e. immune system. "Capacity of insect body to resist all types of microorganisms and their products (Toxins) that tend to damage tissue or an organ is called "immunity". In other words insect immune responses comprise of Cellular responses and Humoral responses both of these responsesare complimentary and may be seen in response to same infection.

Cellular immune response

It is also called as haemocytic immune response. The circulating cells are regularly found in insect haemolymph , called as haemocyte s. These are involved in confirming cellular or haemocytic immunity to insects. The haemocytes are formed in specialized haemopoietic organs. For example, in cricket, *Gryllus* sp., haemopoietic organ is well developed and located at 2nd and 3rd abdominal segments along either side of heart. In other insects, these are as amorphous clumps of cells irregularly distributed along the dorsal vessel.

Classification of haemocytes is contravention still in general haemocytes are classified as Prohaemocyte , Plasmatocytes, Granulocytes, Spherulocytes, Oenocytoides and Adipohaemocytes

1. Prohaemocyte (PRs) are thought to be the stem cells from which all the haemocytes arise.
2. Plasmatocytes (PLs) are larger cells involved in major immunological actions like phagocytosis and nodule formation. In general (PLs) are more than other haemocytes in insect haemolymph .
3. Granulocytes (GRs) are characterized by the presence of many intracellular granules. There are structure less and structured granules. Granulocytes are the major players in encapsulation.
4. Spherulocytes (SPs) are involved in silk formation, melanization, secretion of some haemolymph proteins and clotting response to wound.
5. Oenocytoides (OEs) contribute to the final stage of cuticle formation. They are also responsible for recognition of "non-self".
6. Adipohaemocytes (ADs) are large spiracle or oval cell, the cytoplasm of theses contains characteristic small to very large fat droplets.

Arnold (1974) has concluded that haemocytes do not function in direct defenses against toxicant. Haemocytic defense responses occurred immediately on invasion of microbes and immune functions as fallows were observed Phagocytosis, Nodule formation, Encapsulation and Prophenoloxidase system.

Phagocytosis involves process of engulfing the foreign invaders. Nodule formation is the aggregation of haemocytes that entrap invading microbes. Nodule formation considered

being more important than phagocytosis The Granulocytes initiate nodule formation followed by plasmatocytes. Bacteria, viral polyhedrons, fungal spores and protozoa's are taken care in nodules. Eicosaenoid are responsible for recognition of bacterial infection and the haemocytic response that follows.

Encapsulation take place when an organism or clumps are too big to phagocytosis or to form nodules. It occurs by multilayered aggregation of haemocytes by releasing coagulum and forming envelop around foreign objects, and melanin is deposited in inner layer of the capsule near the surface of the foreign object. Formation of capsule initially restricts growth of offending organism and may result in its death.

Prophenoloxidase System, the enzyme, phenoloxidase (PO) initiates melanization, is ubiquitous in arthropod haemolymph . It occurs as an inactive precursor that is activated by limited proteolysis. After recognition, of the microbes haemocytes are activated for phagocytosis, nodulation and encapsulation. After recognition of foreign invader haemocyte response may differ. All small particles {like single bacteria} are phagocytized, large particles like virus polyhedra and fungal spore required nodule formation. Other large particles like clumps of bacteria elicit multiplayer encapsulation.

PO is key enzyme for oxidation and during oxidation; quinines are formed in the melanize cells surrounding the encapsulated microbes. These quinones are thought to be cytotoxic and facilitate the killing of encapsulated microbes (Soderhall, 1981).

In insects and crustaceans, the prophenoloxidase activation system is an important part of the host defense where it functions to detect and kill invading pathogens, as well as to synthesize melanin for wound healing and encapsulation of pathogens (Soderhall *et al.*, 1998).

Phenoloxidase (PO) also known as tyrosinase, is a bifuntional enzyme possessing both monophenol monooxygenase activity (E.C.1.14.18.1 tyrosine, dihydroxy phenyl alanine, oxygen, oxidoreductase) and *o*-diphenoloxidase activity (E.C.1.10.3.1.o-diphenol, oxygen, oxidoreductase). It is responsible for initiating the biosynthesis of widely distributed melanin pigment in nature (Prota 1992). In addition to melanization of cuticle 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 a*l., 1996; Sugumaran,1998) (2) encapsulation and melanization

of foreign organisms observed as a defense reaction (Ashida and Brey,1995; Gillespie *et al.*, 1997;Soderhall *et al.*,1990) (3) wound healing (Lai-Fook,1966; Sugumaran,1996). In the first process, PO generated 4-alkiquinones serve as sclerotizing agent for quinone tanning reaction-one of the mechanism by which the insect cuticle is hardened to protect the soft bodied insect (Andersen *et al.*,1996). The process of hardening involves the development of cross linked between protein chain and is known as sclerotization (Chapman, 1998). The reaction of quinone methides and quinone methide imine amides with cuticular structural proteins and chitin result in the hardening of the cuticle (Sugumaran, 1998).

In second process, PO serves as a terminal component of an elaborate defense mechanism. Parasites and pathogens which are too big to phagocytosed are found to be usually encapsulated and melanized in insect blood by the action of phenoloxidase (Ashida and Brey, 1995; Gillespie *et al.*,1997; Soderhall *et al.*,1990; Sugumaran,1996). This process not only limited the growth and development of the foreign object but also prevented the damage it can cause to host by creating a physical barrier. Phenoloxidase also provide the cytotoxic quinonoid compound to kill the invading microorganism (Sugumaran, 1996;Nappi and Sugumaran, 1993). Finally during wounding continuous loss of haemolymph is prevented by the rapid deposition of melanin polymer at the wounding site (Lai-Fook,1966; Sugumaran,1996).

Melanization is a common defense response of insects to injury and infection by pathogens and parasites. This reaction is mediated by Phenoloxidase, an enzyme normally present in its inactive from in haemolymph , cuticle and haemocytes (Trudeau *et al.*2001). The insect melanization reaction therefore produced the antiviral activity found in the TBW haemolymph . Thus the innate immunity to viruses that was found here was due to the insect melanization reaction Ourth and Renis (1993).

Anderson *et al.*, (1990) tested immune response in *Trichoplusia ni* challenged with bacteria or baculoviruses and found that an injection of *Autographa californica* nuclaer polyhedrosis virus did not cause an induction any larger than that caused by an injection of Ringer saline. No antibacterial response was observed at all when the viruses were given orally. The ability to melanize is inhibited to various degrees depending on the elicitor; for a short time by a Ringer injection and for progressively longer periods by bacteria and baculovirus injections. The ability to melanize is ultimately restored in virus-infected larvae

as judged from the blackening of dying animals. They also observed a similar inhibition in animals infected with virus. The suppression of the haemolymph 's ability to melanize was noted in larvae injected with baculovirus or bacteria and to lesser extent with Ringer's solulation.

Ourth and Renis (1993) performed experiment on antiviral melanization reaction of Tobacco budworm *Heliothis virescens* haemolymph against DNA and RNA viruses in vitro.with an objective to understand antiviral defense mechanisms in insect. Normal Tobacco bud worm haemolymph collected in EDTA-citrate buffer to inhibit phenoloxidase and the melanization reaction also showed no antiviral activity against HSV-1 and VSV and no . phenoloxidase activity. Activation of haemolymph melanization reaction by phenoloxidase was a necessary prerequisite for antiviral activity to occur. Inhibition of Phenoloxidase and the melanization reaction eliminate the insect antiviral activity.

Phenoloxidase generally are activated by a series of proteases that cleave Prophenoloxidase into its active form however, some Phenoloxidase is also activated by the fatty acids and phospholipides generated by cellular damage. Activated Phenoloxidase generate highly cytotoxic quinines that can inactivate viral pathogen (Trudeau *et al*.,2001).

Lee *et al.* (2000) studied how the activated phenoloxidase from *Tenebrio monitor* larvae enhance the synthesis of melanin by using a vitellogenin- like protein in the presence of dopamine and stated that One of the biological functions of activated Phenoloxidase is synthesis of melanin around invaded foreign material. However, little is know about how activated Phenoloxidase synthesized melanin at the molecular level. Even though it has been suggested that the quinone derivatives generated by activated Phenoloxidase might use endogenous protein components for melanin synthesis in arthropods, there is no report of protein component engaged in melanin synthesis induced by activated Phenoloxidase. In this study, they isolated and characterized protein involved in melanin synthesis, prophenoloxidase activated solution specifically showing Phenoloxidase activity in the presence of Ca^{2+} and 1-3 β glucan, from the haemolymph of larvae of the coleopteran *Tenobrio molitor* by using a Sephadex G-100 column. When G-100 solution was incubated with dopamine to induce melanin synthesis in presence of Ca^{2+} and 1-3 β glucan, four types of protein (160 kDa, prophenoloxidase, Phenoloxidase and 45 kDa) disappeared from SDS PAGE under reducing condition, three protein (160 kDa, Phenoloxidase, 45 kDa) did not

disappear. Furthermore, When the purified 160-kDa melanization-engaging protein was added to a G-100 solution deficient in it, melanin synthesis was enhanced compared with the same solution without the protein. These data support the conclusion that the 160kDa vitellogenin-like protein is involved in arthropod melanin synthesis.

2.2.2 Phenoloxidase in relation to age related resistance, infection by microorganisms and coloration

Miranpuri *et al.*, (1992) studied changes in haemolymph of the migratory grasshopper, *Melanoplus sanguinipes*, infected with an entomopoxvirus. Second instar grasshoppers were inoculated orally with MsEPV (5.0×10^3 occlusion bodies/individual) and haemolymph was collected at specified intervals from 0 to 32 days post-inculcation. Mature OBs were decreased in infected versus control grasshopper after 10-15 days p.i. Phenoloxidase activity initially was at peak at 4 day post inoculation in infected grasshopper and then declined to control level until late in infection. The changes noted in *M. sanguinipes* haemolymph were a direct result of virus infection or a physiological response secondary to infection

Hung and Boucias (1996) studied the phenoloxidase activity in haemolymph of naive and *Beauvaria bassiana* infected *Spodoptera exigua* larvae. They found that, the majority of *S. exigua* haemolymph Phenoloxidase (PO) activity is located in the haemocytes than plasma of naïve larvae. Mid sixth instar larvae possessed 0.2 ± 0.5 and 3.4 ± 2.6 units (1unit = $\Delta 0.001$ A^0 /min at 490 nm) of PO activity/µl of haemolymph equivalent in the plasma and haemocytes lysate (HL) fraction, respectively. The PO titer in HL and plasma fractions sampled from sixth instar larvae during the feeding period (0-60hr) was not significantly different. However, a 10x increase in the PO activity was observed in plasma sampled form the wandering and prepupal stages. They conducted series of assays was conducted to monitor the PO activity in *Beauvaria bassiana* infected *S. exigua* larvae. At 24 hr postchallenge the PO titer in plasma and HL fraction from infected larvae were comparable to the PO titer in fractions from saline controls. Analysis of PO activity reveled that by 48-60 hr postchallenged the PO titer in HL sampled from infected larvae decreased seven fold, whereas plasma PO titer increased.

Nigam *et al.*, (1997) determined the Phenoloxidase activity in the haemolymph of tsetse flies, refractory and susceptibility to infection with *Trypansoma brucei rhodesienes* and found that many defense reaction exhibited by insects are mediated by phenoloxidase enzyme (PO), which normally exists in the haemolymph in an inactive form prophenoloxidase (PrOPO). PrOPOis activated to PO by various microbial factors triggering an enzyme cascade, which culminates in immobilization of pathogen making it more susceptible to other host defenses. An investigation to detect PO in the haemolymph of tsetse flies revealed the presence of an elicititable enzyme system. Levels of spontaneous PrOPO activation in the haemolymph of refractory *Glossina palpalis palpalis* were always higher than those obtained from susceptible *G. morsitans mositans* ($P < 0.001$ at 2& 4 h).

Reeson *et al.*, (1998) reported that the baculovirus resistance in the *Spodoptera* was phenotypically plastic and responding to population density. They found the larvae reared in crowed were black by the third instars and those reared in isolation develop green/brown coloration .The susceptibility to NPV vary considerably with both larvae colour and density across the range of doses of NPV used, overall mortality was 70% for green solitaries compared with 59% for black solitaries and 42% for crowed reared larvae. Phenoloxidase activity in the haemolymph varied according to the colour of the larvae from which it was extracted,Po activity was significantly higher in solitary black and crowded (black) than in green larvae. This suggested that the higher Phenoloxidase level associated with cuticular melanization in crowded-reared larvae might be responsible for the differences in resistance between the phases.

Gillespie *et.al.*, (2000) carried out an experiment to study the immune response of the desert locust *Schistocera gregaria* during mycosis of the entomopathogenic fungus, *Metarhizium anisopliae* var *acriduml.* They found that, topical application of *M.anisopliae* var *acridum* to the desert locust *S. gregaria* resulted in changes in the biochemistry and antimicrobial defense of the haemolymph . *M. anisopliae* var *acridum* colonized the host haemolymph from two day post application. The haemocytes did not attach to, phagocytes or nodulates elements of the fungus. However, the presence of the fungus appeared to stimulate haemocytes aggregation over the first few days of mycosis though the number of aggregates declined subsequently. The total haemocytes count increased two days after application indicating an overall stimulation of the immune system, but declined to a value

below that for uninoculated controls by day four. The differential haemocyte count showed that the initial increase in total haemocytes count was primarily due to a large number of coagulocytes. After day two consistent declines in cell number were observed for all haemocyte classes in mycosed insects. The activity of the enzymes, Phenoloxidase, decreasesd during the course of infection. Phenoloxidase levels in mycosed insects were constantly lower than in control and decline over the period of infection. However, the converse was true for prophenoloxidase. There was significant inverse correlation between PO activity and PPO activities when data from mycosed and control insects were combined. Interestingly there is significant correlation between lysozyme (antibacterial enzyme) and PO.

Jayachandran *et al.*, (2000) studied the age-dependent changes in phenoloxidase activity in *Spodoptera litura* associated with maturation resistance to NPV. They reported that, the phenoloxidase system appeared to have an important role in supplementing the gut barrier by retarding secondary NPV infection (by the virus that manage to gain entry in the haemolymph) in *S.litura*. The cellular haemolymph fraction had a high level of Phenoloxidase activity, which was probably transferred to the plasma in response to NPV infection in the later stage. The PO activity of the plasma fraction has an increasing trend in 5 and 10- day-old larvae in response to NPV treatment. However, the magnitude of PO activity was greater in 10-day-old larvae. The PO activity in plasma in general, was enhanced with the age even without microbial activation. However, the magnitude of increase grows with NPV treatment.

Kalia *et al.*, (2001) studied changes in haemolymph constituents and Phenoloxidase (PO) activity of American bollworm, *Helicoverpa armigera* (Huber) infected with nucleopolyhedrovirus The haemolymph of NPV treated larvae melanized slowly particularly in old larvae. The haemolymph of NPV – treated larvae showed higher PO activity than control particularly in the late stage of the larvae. The haemolymph of NPV-treated larvae showed about 1.5 fold PO activity over that of control in both set of experiments involving 5 and 10-day-old larvae. The plasma PO activity was quite high in NPV-treated larvae as compared to control. However; the cellular PO activity of 10 day old larvae was less than that of control suggesting disintegration of haemocytes and release of PO into plasma. This was correlated with decreased total haemocyte counts (THC) before pupation. There was

negative correlation between relative proportion of granulocyte (GR) and oenocytoid (OE) in THC and the total PO activity. The similar trend was also found in NPV-treated insect. The higher PO activity in NPV treated insect over control may have come from the source of synthesis other than GR and OE.

Trudeau *et al.*, (2001) compared the role of haemocyte pathogenesis in *Heliothis virescens* (Fabricius) and *H. zea* due to *Autographa California* Nucleopolyhedrosis virus (Ac*M*NPV) and found that *H. zea* larvae were highly resistant to virus than those of *H. virescens.* Further, to determine whether, Ac*M*NPV infection triggered the systematic activation of the host Phenoloxidase cascade, in addition to localized tracheal cuticular melanization, they measured and compared haemolymph Phenoloxidase activity of infected *H. virescens* and *H. zea* larvae at 8, 16, 24 and 72 hours post inoculation (hpi). It revealed no difference in the level of Phenoloxidase activity from 8 to 24 hpi. However, at 72 hpi 20% of infected *H. zea* larvae exhibited unusually high level of haemolymph Phenoloxidase activity as compared *H. virescens.*

2.2.3 Characterization of phenoloxidase

Ashida (1971) carried out the purification and characterization of pre- phenoloxidase from haemolymph of the silkworm *Bombyx mori* and also the stability of pre-phenoloxidase and found that at temp 40^0 C pre-phenoloxidase retained its original activity for 60 min whereas at 50^0C 20% of the activity diminished for 60 min and above 55^0C the protein was abruptly inactivated. The pH stability study indicates that the pre-phenoloxidase was stable between pH 5.8 and 9.0 whereas below pH 5.8 or above pH 9.0 inactivation of protein took place.

Pye (1974) in an experiment on microbial activation of prophenoloxidase from immune insect larvae stated that a layer of melanin is frequently observed to be deposited on parasites by insect as a part of their resistance but by blocking quinine formation with phenylthiourea, an insect parasite resistance can be suppressed. He also found that the K_m of Phenoloxidase activated by zymosan and α- chymotrypsin for 4-methylcatechol were similar, 1.20 and 1.07 mM respectively.

Pye (1978) studied activation of prophenoloxidase and inhibition of melanization in the haemolymph of immune *Galleria mellonella* larvae and found that initial Phenoloxidase

activity was located primarily in the cellular fraction. The immune cellular fraction had significantly lower PO activity than the normal cellular fraction. PO activity was uniformly low in both normal and immune plasma fraction. The data on Lineweaver-Bruk plot of initial velocity vs. substrate i.e. 4-methylcatechol indicated an almost identical apparent Km for PO from both the immune and normal cellular fraction, 1.49 and 1.56 mM 4Mc respectively. The immune cellular fraction had a lower V_{max}.

Ashida and Dohke (1980) studied activation of pro-phenoloxidase by the activating enzyme of the silkworm, *Bombyx mori*. It was found that phenoloxidase had different kinetic properties and substrate specificity depending on the pH at which activation took place. Effect of substrate concentration on the enzyme activity was examined with DOPA as a substrate. The three enzyme preparation gave similar apparent K_m values: 1.7 mM, 1.8 mM and 2.0 mM for Phenoloxidase at pH 6.5, pH 7.5 and pH 9.0, respectively. In contrast, these preparation gave much different V_{max} values: being for Phenoloxidase at pH 6.5, pH 7.5 and pH 9.0 were 100,112, and 49, respectively. Since K_m values of Phenoloxidase were similar (1.7-2.0mM) difference in specific activities of these Phenoloxidase preparations should be ascribed to different V_{max} values for these enzymes.

Barrett and Andersen (1981) found three different Phenoloxidase from the cuticle of the mature larvae of the blowfly *Calliphora vicina* and purified it. Enzyme A a typical tyrosinases and Enzyme, B and C are laccases. Substrate specificities for the three enzymes were studied and K_m values were determined for enzyme B with several substrate. The results on Catechol (1.33mM) 4-Methylcatchol (0.22mM) 3,4 dihydroxipheylacitic acid (1.67mM) *N*-aetyldopamine (0.53mM) hydroquinone (0.88mM) and Methylhydroquinone (0.20mM). Inhibitor sensitivities showed that thiourea and phenythiourea as well as a variety of chelating agents have little effect on enzyme B, whereas sodium azide, NaF and NaCN was all inhibitory. In contrast, enzyme A was strongly inhibited by thiourea and phenythiourea but less so by sodium azide and NaF. Enzyme C differs from Enzyme B in being partly inhibited by thiourea, not inhibited by sodium azide and NaF pH activity showed that although the optimum pH for enzyme A appears to be about 7, the enzyme retains nearly maximum activity towards dopamine at pH values between 6.6 - 7.7. In contrast enzyme B is almost totally inactive at these pH values with an optimum at about pH 4.5 whereas enzyme C is inactive at this pH and has an optimum that appears to be above pH 7. All three enzymes

are relatively thermostable but enzyme A appears to be less so than either enzymes B or C. Enzyme B retained 60% and enzyme C about 100% of maximum activity after heating to 80^0C for 5 min, whereas enzyme A lost almost all activity after such treatment.

Taukamoto *et al.*, (1986) isolated and purified latent phenoloxidase from the prepupae of the housefly, *Musca domastica vicina* Maquart. It was found that, the existence of 12.5 mM EDTA in the extraction buffer completely inhibited the activation of latent Phenoloxidase. The latent Phenoloxidase was stable at temperatures between 0 to 40^0C, whereas it was fairly unstable at temperature higher than 50^0C and lost 80% of its activity at 60^0C. There was no spontaneous activation of latent Phenoloxidase at the range of temperature tested here. The latent Phenoloxidase was stable only around pH 6.0. At pH 5.0 the remaining activity was about 25%. At pH 8.0 the remaining activity was more than 30% but was accompanied by the slight activation.

Anderson *et al.*, (1989) purified prophenoloxidase from *Hyalophorcea cropia.* Four proteins were directly involved in its activation, Active phenoloxidase is elicited by the addition of factor C1 and serine protease SP II. Inhibitor I block's the activation with SP II and factor C 1. The characterization of PPO showed a bell shaped dependence on EDTA and EGTA concentration with activation below and inhibition above 2mM. The activity could not be restored by addition of Ca^{2+}, Mg^{2+}, Cu^{2+}. The pH optimum for SPII using the synthetic substrate S-2337 was at 7.8. Phenoloxidase was also assayed for its ability to oxidized L-DOPA over a pH range of 4.0-9.5. Above pH 5, activity of PO was constant and below that, the activity decreased.

Phenoloxidase activity of Acridid grasshopper from the subfamilies Melanoplinae and Oedipodinae was studied by Bidochka *et al.*, (1989) and found that most of the phenoloxidase activity was detected in the plasma fraction of grasshopper, whole-body homogenates and supernatant fraction of the haemolymph. The species representing the Oedipodinae had 20-50% higher percentage of the total phenoloxidase activity associated with particulate matter from a whole-body homogenate when compared to the Melanoplinae. Phenoloxidase activity could not be detected in sclerotized cuticle of adult grasshopper. The phenoloxidase existed as a zymogene, which could be activated by chymotrypsin and inhibited by cyanide compound KCN and NaCN but chelating agent EDTA did not inhibited phenoloxidase activity. The phenoloxidase had optimum activity at 37^oC and its optimum pH

was at 7.3. In order to assess the size difference between the activated (chymotrypsin treated) and non-activated prophenoloxidase, they performed native PAGE on *M.sanguinipes* haemolymph samples. The results of staining the gel with DOPA shown that Chymotrypsin-activated haemolymph had two bands of phenoloxidase activity as opposed to one band in the non-activated haemolymph. The additional band in Chymotrypsin-activated haemolymph sample migrated further in the native gel, suggesting cleavage of Prophenoloxidase to an active smaller Phenoloxidase.

For isolation and characterization of haemolymph phenoloxidase Lockey and Ourth (1992) isolated Phenoloxidase from haemolymph of *H. virescens* larvae and found that its molecular weight was approximately 250 kDa. The characterization of haemolymph phenoloxidase was done. The optimum pH and the optimum temperature for Phenoloxidase activity were 9.0 and 45^{0}C respectively. Whereas the enzyme kinetics study revealed that Phenoloxidase had a K_m of 2.25 and V_{max} of 0.235 Δ A /min/mg. Similarly the inhibitor study of enzyme showed that, the activity of phenoloxidase was not affected by calcium or EGTA, but the activity was inhibited by both EDTA and SDS.

Aspan *et al.*, (1995) in a experiment on cDNA cloning of prophenoloxidase from the freshwater crayfish *Paifastacus leniusculus* and its activation purified and cloned Prophenoloxidase (proPO) (an enzyme that is the terminal component of the so called proPoO activating system, a defense and recognition system in crustaceans and insects). Further they purified the pro PO from the cryfish blood cell cDNA library and used it to test the ability of the enzyme to oxidize different substrates (Mono- and diphenols). The enzyme readily hydroxilates the monophenols tyramine and tyrosine. The enzyme oxidizes o-diphenols, such as dopamine N-aetyldopamine, L- dihydroxyphenyalanine, 4-methylcatachol and catechol but did not oxidize the two P-phenols tested gentisic acid and hydroquinone. Thus cryfish ProPO has substrate specificity similar to other tyrosinases. The substrate specificity of the crustaceans-phenoloxidase very similar to the insect tyrosinases. In addition, inhibitors that typically inhibit tyrosinases, such as diethyldithiocarbamate, phenylthiouria and 4- nitrocatechol were effective in inhibiting the L-dihydroxyphenyalanine oxidizing activity of cryfish proPO.

Hall *et al.*, (1995) studied the proenzyme of *Manduca sexta* phenoloxidase, its purification, activation, substrate specificity of the active enzyme, and molecular cloning.

They isolate Phenoloxidase proenzyme from the haemolymph of *M. Sexta* larvae and purified to homogeneity. During its characterization the substrate specificity of Cetylpyridirium chloride (CTC) activated proPO, N- β- Alanyldopamine and 4-methylcatechol proved superior substrate and DOPA, often used as a substrate for routine assay of insect PO, proved to be poor substrate for *M. Sexta* haemolymph PO. In the light of these results, routine use of DOPA as a substrate for the enzyme is not recommended.

Hung and Boucias (1996) studied the characterization of haemolymph phenoloxidase, which revealed that the *S. exigua* HL PO had a pH Optimum of 7.0 when assayed against DL-Dopa. The relative activity of haemocyte lysate (HL) PO was not inhibited by addition of EGTA or EDTA.

Chosa *et al.*, (1997) In an experiment on activation of Prophenoloxidase A_1 by an activating enzyme in *Dorsophila melanogaster*, isolate an activating enzyme for prophenoloxidase A_1 from pupae of *Dorsophila melanogaster*. The activation and purification of prophenoloxidase A_1 with this enzyme was analyzed. The activation reaction on proPO A_1 concentration was examined over the range of 0.4-1.0 mg protein /ml. Double reciprocal plots of the reaction velocity and the substrate (L-DOPA) concentrations .K_m value was calculated to be 0.51mg proPO A_1 /ml. The molar extinction coefficient of proPOA_1 is calculated to be $1.0 \times 10^5\ M^{-1}cm^{-1}$.based on this value K_m for proPOA_1 is' considered to be around 3.0×10^{-6} M.

Chase *et al.*, (2000) performed purification, characterization and molecular cloning of prophenoloxidase from *Sarcophaga bullata* and stated that Prophenoloxidase (PPO) was a key enzyme associated with both melanin biosynthesis and sclerotization in insects. This enzyme is involved in three physiologically important processes viz., cuticular hardening, defense reaction and wound healing in insect. It was isolated from larval haemolymph of *Sarcophaga bullata*, and purified by employing ammonium sulfate precipitation. Enzyme exhibited two closely related bands on 7.5% SDS-PAGE under denaturing condition. The active enzyme exhibited wide substrate specificity. N-acetyldopamin, N-β-alanyldopamine, and dopamine proved to be far better substrate for the enzyme than dopa . The activated PO exhibited a typical bell shaped pH curve with optimum activity observed at about pH 7.

Nagai and Kawabata (2000) in an experiment on a link between blood coagulation and prophenoloxidase activation in arthropods host defense found that Phenoloxidase, a

copper containing enzyme was widely distributed not only in animal but also in plant and fungi, which was responsible for initiating the biosynthesis of melanin. Activation of prophenoloxidase was important in defense. Here they show that the coagulation cascade of horseshoe crab was linked to prophenoloxidase activation like other arthropods. Phenoloxidase the functionally transformed subunit showed an o-diphenol oxidase activity. 4-Methylcatechol and catechol were readily oxidized, but tyrosine and hydroquinone were not significantly oxidized. Typical inhibitors for arthropods phenoloxidase, such as phenylthiourea (12.5mM) and EDTA (25 mM) competently inhibited the activity of enzyme.

Pro-phenoloxidase (PPo) in insect is implicated in the defense against microbes and wounding . The presence of PPo in the cuticle was suggested more than 30 year ago, but it has not been purified . The extract of the cutical of the silkworm, *Bombax mori*, was shown to contain two PPO isomers (F-types and S-types).The two isomers were purified to homogeneity form haemolymph of the same insect . The isomers in the haemolymph and cuticle were activated by specific activating enzymes. The resulting active Phenoloxidase exhibited almost the same substrate specificities and specific activity towards O-diphenol. The phenols were found to serve as better substrates in the order Dopamine, N-aetyldopamine, L-Dopa, methylhydroquinone and hydroquinone. Activities of POs were inhibited almost completely by phenythiourea at a concentration of 1uM in the oxidation of any of the substrates used (Asano and Ashida, 2001)

2.2.4 Location of phenoloxidase

Leonard *et al.*, (1985) studied prophenoloxidase and protease activity of *Blaberus craniifer* haemocytes and attempts were also made to determine whether the proPO system was cell-associated and/or wholly or partially present in the plasma. Results indicated that most of the ProPO system is soluble as the PO activity was present in the 2800 and 70,000 *g* supernatants (HLS preparations) and less associated with membrane in the pelleted debris. The plasma contained minimal activity but this may be partly related to the use of the anticoagulant (AC) which was shown to inactivate 50 % of the PO activity of the HLS preparation, even after removal of AC by dialysis. This indicates that the haemocytes are the main repository for proPO. Secondly it was possible that proteases were involved in activation of proPO. It was also observed that the protease inhibitors, benzamidine, STI and

pNPGB could block laminarin activation of Blaberus proPO indicating that the activating enzyme is a serine protease.

Iwama and Ashida (1986) studied biosynthesis of prophenoloxidase in haemocytes of larval haemolymph of the silkworm, *Bombyx mori.* Polyclonal antibodies against silkworm prophenoloxidase were prepared and used to study the biosynthesis of prophenoloxidase. When fat body integument and haemocytes of silkworm larvae were cultured in presence of methionine, only the haemocytes incorporated radioactivity in to prophenoloxidase and released it in to the culture medium. Among the haemocytes stained with FITC-label anti-prophenoloxidse antibody, only the oenocytoides fluoresced under florescence microscopie. These results indicated that prophenoloxidase was synthesized by the oenocytoides. Prophenoloxidase must first be released from the haemocytes and then transported through the plasma to the site were the enzyme as activated. This implies that prophenoloxidse is released from oenocytoides *in vitro and in vivo.* Thus, prophenoloxidase is likely to be present in both plasma and oenocytoides under physiological conditions.

2.2.5 Immunosuppression or inhibition of phenoloxidase

Azambuja *et al.*, (1991) studied immune depression in *Rhodnius prolixus* induced by the growth inhibitor Azadirachtin. It was found that azadirachtin (1.0μg/ml) if fed to last instar larvae of *Rhodnius prolixus* through a blood meal, affect the immune reactivity; as shown by a significant reduction in numbers of haemocytes and nodule formation, following challenge with *Enterobacter cloacae* B12. A reduction in ability to produce antibacterial and Lysozyme activities in the haemolymph, when inoculated with bacteria and a decreased ability of Azadirachtin-treated insect, to destroy the primary infection caused by inoculation of *E. cloacae* cells. However, the experiment, unlike other immune reactions, failed to demonstrate any interference of azadirachtin with the prophenoloxidase-activating system since the melanin production was not reduced when this system was stimulated by trypsin or by the presence of bacteria in the haemolymph suggesting that the immune response was deficient in the azadirachtin treated insects.

Parkinson *et al.*, (2001) identified Phenoloxidase (PO) activity among several biologically active factors in venom from the parasitoid wasp *Pimpla hypochondriaca.*. For the first time reported PO as a venom constituent and it invites speculation as to its function.

Although the mode of action of PO products generated from the enzyme derived from haemocytes was not fully understood. They studies have identified a protein located in haemocytes plasma membrane, which participates, with PO in a process that entrap bacteria. This provided that PO products could bind to the haemocytes plasma membrane. Damage to haemocyte's, including lysis and degeneration, has also been attributed to their over stimulation by PO products. They opined that venom PO derived product if produces in sufficient quantity in vivo could similarly bind to haemocytes and disrupts their function, thus contributing to host immune suppression.

Mantawy and Mahmoud (2002) investigated the effect of feeding of *Allium cepa* and *Allium sativum* on glucose, glycogen, and protein bands profile and phenol oxidase activity in *Biompha alexandrina* after 1, 2, 3 and 7 days. Different protein bands were separated from the control and treated snails' haemolymph after 24 hours and one week by using SDS-electrophoresis. The study revealed that glucose and glycogen were decreased significantly after feeding on onion and garlic. Also 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. Data obtained from SDS-electrophoresis showed variations in the different protein bands reflect the enzymatic changes in the snails' tissue. So the snails' fecundity may be reduced and in turn disturb the life cycle of schistosome parasite.

Goldsworthy *et al.*, (2003) made investigation on an interaction between the locust (*Locusta migratetoria*) endocrine and immune systems in vivo in relation to nodule formation and activation of the prophenoloxidase cascade in the haemolymph. Injection of bacterial lipopolysaccharide (LPS) extracted from *Escherichia coli* induced nodule formation in larval and adult locust but have not increased Phenoloxidase activity in the haemolymph . Co-injection of Adipokinetic hormone- I (*Lom*-AKH-I) with LPS stimulated greater numbers of nodules to be formed in larval and adult locusts and activated phenoloxidase in haemolymph of mature adults but not of nymphs. Remarkably detoxified LPS activated phenoloxidase in the absence of *Lom* –AKH-I, although co-injection with hormone dose enhanced this response Co-injection of a water-soluble inhibiter of eicosaenoid synthesis, diclofenac(2-[(2.6-diclorophenyl)amino]benzeneacetic acid)reduced nodule formation in response to injection of LPS (both in the absence and presences of hormone) in dose –

dependent manner, however it has not prevent activation of Phenoloxidase in adult locust. It was shown that nodule formation and activation of the prophenoloxidase in locust haemolymph can both be enhanced by *Lom*-AKH-I, but it was argued that these processes involved distinct mechanism in which eicosaenoid synthesis is important for nodule formation, but not for the increased phenoloxidase activity.

Mullen and Goldsworthy (2003) studied the changes in lipophorins related to the activation of phenoloxidase in the haemolymph of *Locusta migratoria*. They reported response to injection of immunogens, was age –dependent, being absent in fifth nymphal stage and newly emerged adults, but only becoming evident four days after the final molt. This pattern of change in Phenoloxidase activation correlated with pattern of change in the concentration of apolipophorin–III (apoLp-III) in the haemolymph .Further they reported that injection of a conspecific adipokinetic hormone (*Lom*- AKH-I) has no effect on the Phenoloxidase response in nymphs or newly emerged adults but, was observed in adult older than four days. Co-injection of the hormone with laminarin prolongd the activation of phenoloxidase in the haemolymph . A similar enhancement of the response to laminarin was observed in locusts those have been starved for 48 hr but not injected with AKH-I. During most of the fifth stadium, injection of laminarin resulted in a decreased level of prophenoloxidase in the haemolymph; an effect that was not observed in adult of any age. Marked changes in the concentration of apoLp-III, and the formation of LDLp in the haemolymph, were observed after injection of laminarin (LPS) and these were remarkably similar, at least qualitatively, to those that occur after injection of AKH-I.

Adamo (2004) examined the relationship between two common estimates of insect immunocompetence, Phenoloxidase and lysozyme-like enzyme activity and resistance to three common insect bacterial pathogen, *Serratia marcescens, S. liquefaciens,* and *Bacillus cereus*. There was a correlation between total Phenoloxidase and baseline lysozyme like activity within individual. However, total Phenoloxidase and baseline lysozyme- like activity level did not predict which male crickets would survive any of the three bacterial challenges. Lysozyme-like activity increased after an immune challenge and greater the increase greater the chance that the cricket would survive. The cricket with a greater total haemolymph protein concentration were also more likely to survive a challenge with any of the three bacterial pathogens than the cricket with lower total haemolymph protein concentration.

Garcia *et al.*, (2004) investigated the effects of ecosenoid biosynthesis inhibitors on the haemocytes microaggriegation and prophenoloxidase (proPO) system in the haemolymph, parasitemia and mortality of *Rhodnius prolixus* infected with *Trypanosoma rangeli.* Hemocoelic injection of live *T. rangeli* epimastigotes into fifth- instars larvae of *R. prolixus* those were fed previously on blood containing an inhibitor of phospholipase A_2 (dexamethasone) which is a specific inhibitor of the cyclooxygenase pathway (indomethacin) and a non–selective lipoxygenase inhibitors (NDGA). It reduced microaggriegation, secondly attenuated the proPO in the haemolymph and thirdly enhanced parasitemia and mortality induced by the parasite challenge in these insects. The effects obtained by dexamethasone administered orally were counteracted by inoculation of the insects with arachidonic acid. They suggested that, the infectivity of *T. rangeli* could be increased by interference with the *R. prolixus* immune system.

2.3 Total Haemocyte count and morphological changes in haemocytes of virus infected larvae

Shapiro (1967) studied the pathologic changes in the blood of the greater wax moth, *Galleria mellonella,* during the course of Nucleopolyhedrosis and starvation. In larvae inoculated with nucleopolyhedrosis virus and fed a normal diet, no change was observed in the total haemocytes count (THC) until day 10, when a significant decrease was detected. The THC's increased in control larvae but decreased in starved larvae. Within the first 5 days of the test the THC's in inoculated larvae were significantly higher than in starved larvae but were significantly lower than in control larvae.

Wittig (1968) observed phagocytosis by blood cell in healthy and diseased caterpillar while making some observation concerning virus inclusion bodies. In the THC's of granulosis-infected and control armyworms from 4 days after start of the test. The percentage of prohemocytes was considerably increased over that of control the data suggested concurrence of a sharp drop in the THC's with the appearance of capsule in the which has been attributed to a reduction of the total haemocyte of circulating cells, and there by increase in percentage of prohaemocyte blood during the 1st day after molt to the L5, 10 larvae fed with capsule suspension had mean THC of 20,215 haemocytes/mm^3 whereas 10 control larvae averaged 24,870 haemocytes/mm^3. This 19% decreased was significant at the

5% level. The number of haemocytes per unit volume of blood was greatly reduced during the later part of the disease. Eight days after start of test the DHC of the infected larvae differed from that of the control, as follows macroplasmatocyte count, 23.2% lower, spherule cell count, 15.5% higher and prohaemocyte count, 8.4% higher.

Kislev *et al.*, (1969) performed electron-microscopic studies on haemocytes of the Egyptian cotton worm, *Spodoptera littoralies* (Boisduval) infected with nuclear-polyhedrosis virus as compared to noninfected haemocytes. Electron micrographs of haemocyte s, extracted from caterpillar 1 hr after injection of suspension captaining 4 x 10^7 PIB/ml reveled that about 10% of total cell number were plasmatocytoids containing 1-11 viral polyhedra in the cytoplasm. Of the four major types of haemocytes differentiated in the blood of this insect, virus formation was found to take place mainly in the plasmsmatocytoids and only to a much lesser extent in the granular haemocytes and oenocytoides. Adipohaemocytes were never seen to sustain virus development. Plasmatocytoides were observed phagocytosing free virus particles as well as several whole polyhedra.

Shapiro *et al.*, (1969) examined haemocytes changes in larvae of the bollworm *Heliothis zea* infected with a Nucleopolyhedrosis virus. The average number of circulating haemocytes in both virus-exposed and non-exposed bollworm increased significantly during the first day of exposure. After 3 days of exposure, the average number of haemocytes decreased dramatically in larvae exposed to high virus dose. A similar decrease was not observed in nonexposed larvae or larvae exposed to a low virus dose. The average number of phagocytic haemocytes decreased just prior to pupation in both virus-exposed and nonexposed larvae. The reduction in phagocytes was greater in virus-exposed larvae (63%) than in nonexposed larvae (31%). The presence of inclusion bodies in the nucleus and latex particles in the cytoplasm indicated infected haemocytes to be still phagocytic. Differences between the number of spherule haemocytes in virus-exposed and nonexposed larvae were not evident during the first 3 days of exposure. A steady increase in the average number of spherule cell occurred during maturation of both group of larvae. On 4th day however, the number of spherule haemocytes decreased more than 55% in nonexposed larvae and increased about 25% in larvae exposed to virus.

Jacob and Subramaniam (1974) performed experiment on haemocytes changes in larvae of the Tobacco caterpillar *Spodoptera litura* F., infected with nuclear-polyhedrosis

virus. Haemocytes changes due to infection of nuclear polyhedrosis in the larvae of *Spodoptera litura* are described. Early fourth instar larvae were inoculated with 10^5 polyhedra each. The number of circulating haemocytes (THC) was significantly higher in infected larvae at 24 hour after inoculation. But at 48,72,96, and 120 hour following inoculation, the THC was significantly lower than in the corresponding healthy larvae. Further the THC decreased progressively from 48 hour onwards in infected larvae as against a general increasing trend in healthy ones.

Rabindra and Subramaniam (1974) conducted studies on nuclear polyhedrosis of *Heliothis armigera* (Hbn.) susceptibility and gross pathology. They observed that the average number of haemocytes in infected larvae with nuclear polyhedrosis virus decreased steadily and drastically after 48 hours from 28640/mm^3 to 15085/mm^3 at 120 hours. The reduction of total haemocytes count in *H. armigera* the invasion of the nuclei of blood cells by the virus, as polyhedral bodies could be seen in the nuclei of blood cell by 72 hours post-inoculation.

Lea (1986) studied a *Sericesthis* Iridesdent virus infection of the haemocytes of the Wax Moth *Galleria mellonella* and it's effect on total and differential counts and haemocytes ontogeny. Total and differential haemocytes counts were made to detect gross changes in the haemocytes picture resulting from response of the infection. Total counts were generally lower in infected insect than either in control group. The relative depration of total count in infected group is especially pronounced in the day 12 sample in which the average total count of the infected group was about 50% that of the control group.

Anderson *et al.*, (1990) examine immune responses in *Trichoplusia ni* challenged with bacteria or baculoviruses. All infections were done on day 1st day of last instar larvae. Injections were made on the dorsal side of the fifth or sixth abdominal segment with 4 x 10^5 plaque forming unit (pfu) of AcNPV or with *Enterobacter cloacae* (3 x 10^6 ml^{-1}). Larvae were also infected with orally by giving each larva a piece of food (approx100mg) containing 2 x 10^6 pfu of AcNPV and also with ringer. Determination of total cell number in haemolymph sample obtained from *T.ni* larvae scarified at different time after injection of AcNPV, *E. cloacae* or ringer's solution was made there was rapid drop in cell counts during the first hours following the different injection. The recovery of *T.ni* to normal haemocyte levels was faster for ringer treated followed by the bacteria treated and most slowly with the virus-injected

Miranpuri *et al.*, (1992) studied changes in haemolymph of the migratory grasshopper, *Melanoplus sanguinipes,* infected with an entomopoxvirus. Second instar grasshoppers were inoculated orally with MsEPV (5.0 x 10^3 occlusion bodies/individual) and haemolymph was collected at specified intervals from 0 to 32 days post-inculcation. Mature OBs were decreased in infected versus control grasshopper after 10-15 days post –infection (p.i.). Haemocytes from infected individuals on Day 28 p.i. showed a five-to six fold increase in binding of fluorescein-isothiocyanate labeled wheat germ agglutinin. Haemocytes morphological changes were observed late in infection including a small percentage of enlarged haemocytes and cell with extensive pseudopodia. MsEPV OBs were also observed in the cytoplasm of haemocytes late in infection.

Kalia *et al.*, (2001) studied changes in haemolymph constituents and phenoloxidase (PO) activity of American bollworm, *Helicoverpa armigera* (Huber) infected with nucleopolyhedrovirus. They found six types of haemocytes viz., prohaemocyte (PR) plasmatocytes (PL) granular cells (GR) spherules cell (SP) Oenocytoides (OE) and adipohaemocytes (AP) in the haemolymph of larvae of American bollworm *H. armigera.* They reported that the total and different haemocytes counts (THC and DHC) in *H. armigera* haemolymph were affected by nucleopolyhedrovirus (NPV) treatment. There was a general decrease in THC in response to NPV treatment in both young and old larvae. However the decrease was more apparent in 5 and 8 day old larvae than in 10-day-old larvae. Plasmatocytes and granular cell in 10 day larvae initially phgocytosed polyhedra, however, disintegrated after 3 and 4 hr.

CHAPTER III

MATERIAL AND METHODS

The present investigation was undertaken with the objectives to study the age dependent toxicity of HaNPV to *H. armigera,* role of Phenoloxidase (PO) associated with the age related resistance, the correlation of colour of larvae with the toxicity of HaNPV and phenoloxidase levels in the field collected larvae of *H. armigera, in vitro* inhibition of PO and finally effect of different phenoloxidase inhibitors in combination with HaNPV in the laboratory, also total haemocytes counts in virosed and normal *H. armigera* larvae.

The work was carried out during 2003 and 2004 in the laboratory of Department of Entomology and Insect Biotechnology laboratory, Dr. PDKV, Akola. The material required and the methods followed during the course of present investigation are described below.

3.1 Material

3.1.1 Bioassay of *H. armigera*

3.1.1.1 Mass rearing of *H. armigera*

The material for mass-rearing of *H. armigera* in the laboratory included multicellular trays, transparent plastic vials of different sizes, adult emergence and mating chamber, incubation jar, forceps, fine camel hairbrush, absorbent cotton, muslin cloth, tissue culture blades, test tube, rubber bands, threads, beakers, rearing rack, laminar air flow, semi-synthetic diet (chickpea based) and adult diet, mixture/blender, hot air oven, steel container, glass petriplates, electronic balance, polythene wrapper, refrigerator, OHP marker and thermo-hygrometer, aluminum foil.

3.1.1.2 Bioassay of HaNPV against *H.armigera*

Larvae of *H. armigera* in different instar (i.e. 2nd to 5th) transparent plastic vials, multicellular trays, semi-synthetic diet (chickpea based), HaNPV, micropipette, forceps, rubber bands, test tubes, measuring cylinder etc.

3.1.2 Enzyme assay and characterization

3.1.2.1 Chemicals used

Ethylenediamine tetra acetic acid (EDTA), Phenylthiourea (PTU), Phenyl methyl sulfonyl fluoride (PMSF), Sodium dodecyl sulfate (SDS), Bradford reagent, L-3-4 Dihydroxy phenyl alanine (L- DOPA), DL-3-4- Dihydroxy phenyl alanine (DL-DOPA), L-Tyrosine, Dopamine Hydrochloride, 3-hydroxytyramine chloride (Dopamine), 4-Methylbrenzcatecholin, Dimethyl sulfoxide (DMSO), Soybean trypsin inhibitor (STI), Ethylene glycol-bis (β-Aminoethyl Ether), N, N, N, N-tetraacetic Acid (EGTA), Garlic extract, Phosphate buffer (pH 7), citrate buffer (for pH range 3.0-6.0), Sodium phosphate buffer (SPB) (for pH range 7.0-8.0), Sodium bicarbonate buffer (for pH range 9.0-11.0).

3.1.2.2 Instruments used

Microplate reader (Metertech Σ 960, USA), Thermal cycler (Applid Biosystems 9700), High-speed refrigerated centrifuge (Haurues), Hoefer SE600 slab gel unit (Hofer, San Francisco, CA), Gel Dock System EQ (Biorad), UV spectrophotometer (Hitachi-2001), Microwave (LG), pH Meter (Thermo orion model 420), Electronic balance (Sartorium, Germany), -80^{0}C Deep freezer (Heto Holton, Denmark), micropipettes of various sizes (Qualigens), Refrigerator (Videocon, India.), BOD incubator (Newtonic Instruments, India.), Mixer/blender (Kenstar, France), Laminar air flow (Klenzaid, India), Water filtration assembly (Millipore)

3.1.2.3. Polyacrylamide gel electrophoresis (NATIVE PAGE)

a) Sodium phosphate buffer (0.1 N) pH 7 (SPB)

Sodium phosphate (Na_2HPO_4)	:	3.56 ml
Distilled Water	:	200 ml
Sodium di-hydrogen ortho phosphate (Na_2HPO_4)	:	1.25 ml
Distilled Water	:	80 ml

b) Stock tris-glycine electrode buffer solution (pH 8.3)[10X]

Tris	:	6.0 g
Glycine	:	28.8 g
Distilled water	:	upto1000 ml

c) Working electrode buffer (pH 8.3)[1X]

Tris glycine electrode buffer (pH 8.3)	:	1 part
Distilled water	:	9 part

d) Tris chloride buffer stock solution (pH 8.9) [4X]

HCl 1 N	:	48 ml
Tris	:	36.6 g
Tetra methylene ethylene diamide (TEMED)	:	0.23 ml
Distilled water	:	to100 ml

e) Tris-chloride buffer stock solution (pH 6.7) [4X]

HCl 1 N	:	48 ml
Tris	:	5.98 g
TEMED	:	0.46 ml
Distilled water	:	to100 ml

f) Bis acrylamide monomeric acrylamide solution

Acrylamide	:	28 g
N^1N^1 Methylene bis-acrylamide	:	0.74 g
Distilled water	:	to100 ml

g) Ammonium per sulphate solution (10%), fresh

Ammonium per sulphate	:	0.1 g
Distilled water	:	1 ml

h) Bromophenol blue solution

Bromophenol blue	:	25 mg
Tris-chloride buffer solution (pH 6.7)	:	10 ml

i) Resolving gel solution (6%)

Tris-chloride buffer stock solution (pH 8.9)	:	5 ml
Monomeric solution	:	4 ml
Distilled water	:	10.6 ml
Ammonium per sulphate solution	:	200 µl
TEMED	:	16 µl

j) Stacking gel solution (5%)

Tris chloride buffer stock solution (pH 6.7)	:	0.63 ml
Monomeric solution	:	0.83 ml
Distilled water	:	3.4 ml
Ammonium per sulphate solution (10 %)	:	50 µl
TEMED	:	5 µl

k) Resolving gel solution (10%)

Tris-chloride buffer stock solution (pH 8.9)	:	5 ml
Monomeric solution	:	6.7 ml
Distilled water	:	7.9 ml
Ammonium per sulphate solution	:	200 µl
TEMED	:	8 µl

l) Sample buffer

Tris-chloride buffer stock solution (pH 6.7)	:	25 ml
Glycerol	:	10 ml
Distilled water	:	to100 ml

m) Substrate reagent for staining of PO isozyme

SPB, 0.1 M, pH 7	:	100 ml
Dihydroxy phenyl alanine	:	0.040mg

3.1.3 Haemocytes count

3.1.3.1 Chemicals used

Anticoagulant buffer (Azambuja *et. al.*, 1991)

0.01	M	EDTA
0.1	M	Glucose
0.062	M	Sodium chloride
0.03	M	Trisodium citrate
0.026	M	Citric acid

3.1.3.2 Leishman's stain

0.3 g of Leishman's stain powder was grinded with little amount of absolute methyl alcohol to make even suspension. A total of 200 ml methanol was added to it and aged in dark bottle for few weeks prior to use

3.1.3.3 Instruments used

Tissue culture blade, microcentrifuge tubes of various sizes, micropipette of various volumes, Neubauer's improved Haemocytometer (Marienfeld Germany), Research Microscope (Olympus), glass slides (Blue star).

3.2. Methods

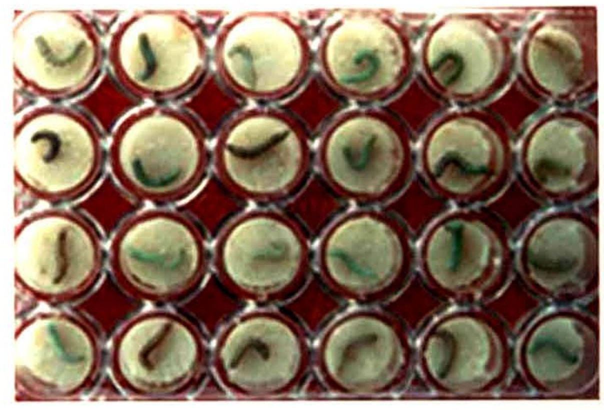
3.2.1 Mass rearing of *H. armigera*

The field-collected larvae from various crops were reared individually in pre sterilized plastic vials to avoid cannibalism. The larvae were provided with semi-synthetic diet (Armes *et al.*, 1992) at regular intervals and they were allowed to pupate in the same vials. The pupae were collected and disinfected with 0.02 per cent sodium hypochlorite solution. Disinfected pupae were sexed (Narayan *et al.*, 1977 and Navrajan Paul *et al.*, 1979) and transferred in adult emergence chamber. The adults emerged were transferred in mating chamber by maintaining a male female ratio (1: 1) and were provided with adult diet (Table 2). Five pairs of moths were released in each mating chamber (Plate1-b), which were prepared by using glass bell jar. The small strips of cotton cloth nappy liners were used as an ovipositional substrate for the female moths. The strips of cotton cloth with eggs were regularly replaced after two days and were kept in small incubation jars for hatching. The neonates emerging from the eggs were transferred with the help of camel hairbrush on the cubes of freshly prepared semi-synthetic diet (Plate 1-a). The larvae from 'F-I' generation were used for bioassay studies (Plate 1-c). *H. armigera* larvae were periodically collected from the field and added to the cultures in order to maintain the vigour of the culture as well as for sustainability of the *H. armigera* culture under laboratory conditions.

3.2.1.1 Procedure for preparation of semi-synthetic diet for *H. armigera* larvae

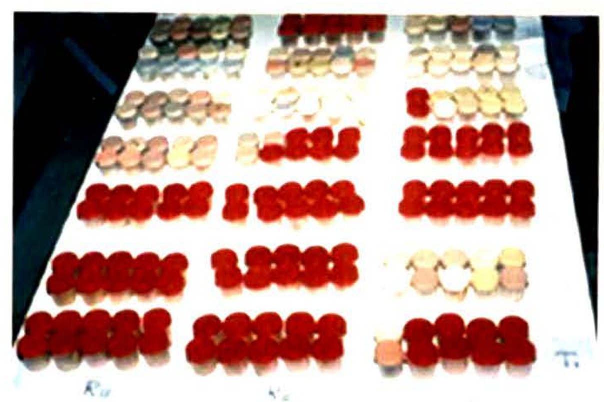
All ingredients of Part-A were mixed with the help of blender in 550 ml of warm distilled water. In another saucepan, 600 ml of distilled water was boiled and yeast granules were added in boiling water and allowed to dissolve completely. During the process of dissolving of the yeast granules, agar-agar was added slowly to the boiling yeast solution to form a homogenous mixture.

This mixture (Part - B) was poured in the content of blender (Part - A) and both the components were blended for one minute. The hot diet was poured in prewashed plastic bottle and dispensed into cell trays and plastic vials to a depth of about 5 mm. After the diet was solidified, the diet trays were stored in refrigerator till further use.



a. Rearing on artificial diet

b. Adult mating chamber



c. HaNPV Bioassay study

Plate 1. Rearing and Bioassay of *H. armigera*

Table 1: Semi-synthetic diet for rearing of *H. armigera* larvae (Armes *et al.*, 1992)

Sr. No.	Ingredients	Quantity
	Part-A	
1.	Chickpea flour	160 g
2.	Wheat germ	60 g
3.	Ascorbic acid	5.3 g
4.	Methyl-4-hydroxybenzoate	3.3 g
5.	Sorbic acid	1.7 g
6.	Aureomycin	2.5 g
7.	Formaldehyde (10% v/v)	13.5 ml
8.	Distilled water	550 ml
	Part -B	
1.	Yeast (Dried bakers/brewers)	53 g
2.	Agar-agar	16 g
3.	Distilled water	600 ml

Procedure for Adult Diet: The distilled water was first boiled and cooled at room temperature. Then all the ingredients (Table 2) were dissolved in it by taking water in a conical flask of liter capacity. The adult diet, thus prepared was stored in sterilized amber coloured bottle in refrigerator.

Table 2: Adult diet for *H. armigera* moths

Sr. No	Ingredients	Quantity
1.	Sucrose	50 g
2.	Honey	50 ml
3.	Aureomycin	2.5 g
4.	Streptomycin sulphate	1 g
5.	E-vitamin capsule	1 g
6.	Methyl-4-hydroxybenzoate	1 g
7.	Formaldehyde (10%v/v)	1 ml
8.	Vitamin stock solution	5 ml
9.	Distilled water	500 ml

Table 3: Composition of vitamin stock solution (Vanderzant, 1974)

Sr.	Vitamins	Quantity
1.	Riboflavin	300 mg
2.	Pyridoxine	150 mg
3.	Thiamine	150 mg
4.	Folic acid	150 mg
5.	Nicotinic acid	600 mg
6.	Calcium phenthoate	600 mg
7.	Biotin	12 mg
8.	Vitamin B_{12}	1.2 mg
9.	Distilled water	1000 ml

3.2.2 Bioassay procedure

The test insects were selected on an age basis and each group was then further selected by weighing the larvae to ensure the larvae within each age group to be roughly of similar weight. One cm^3 size cube of semi-synthetic diet was cut and surface contamination of larval diet was done with 0.2 ml of different concentrations ranging from 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, 2×10^3, 2×10^4, 2×10^5, 2×10^6, 2×10^7 which were prepared by the serial dilution from stock solution of 2×10^9 POB/ml (Commercial formulation of Department of Entomology, Dr. PDKV, Akola). Each treatment was replicated thrice with ten larvae per replication. Larvae of 2nd to 5th instar *H. armigera* after prestarvation of 6 hrs were allowed to feed on HaNPV impregnated artificial diet for 24 hr. A control was also maintained on diet treated with distilled water and similarly was allowed to feed for 24 hrs, subsequently they were transferred to fresh diet on next day. The larvae, which consumed the whole piece of semisynthetic diet, were considered for further study.

Mortality was recorded after every 24 hr till pupation in case larvae survived. The cumulative mortality in three replications was pooled together and corrected percent mortality was calculated by using Abott's formula (Abott, 1925). The data obtained were

subjected to probit analysis, by using the softwares (Indostat and Polo) and LC_{50} values for 2nd to 5th instar larvae were worked out.

3.2.3 Protein estimation

Protein concentration of insect haemolymph was determined by following Bradford method (1976) by using Bovine Serum Albumin (BSA) [Sigma] as a standard protein to construct the standard curve. The Bradford pristine assay is a dye-binding assay in which a differential colour change of a dye occurs in response to various concentration of protein.

Development of standard curve

1) 1 mg/ml solution of BSA was taken in test tubes in different amount ranging from 2µl to 20µl, to make the concentration of BSA from 2 µg to 20 µg. Each concentration was prepared in triplicate.
2) Volume of the protein solution was adjusted to 20 µl by using 0.15N NaCl.
3) 200 µl ready to use Bradford reagent [Amarsham Pharmasia, diluted 1:4 by distilled water] was added in each test tube.
4) Test tubes were incubated for 15 min at room temperature.
5) Absorbance was taken at 600 nm on Microplate reader.
6) Calibrated curve was plotted between mean value of concentration on X-axis and mean value of absorbance on Y-axis.
7) Protein concentrations of enzyme from virosed and normal larvae of *H. armigera* were calculated by taking 5 µl of haemolymph.

Procedure

1) Five microliter of haemolymph solution was added in the well.
2) Then 15 µl of 0.15 N NaCl solution were added in the wells.
3) 200µl of Bradford reagent (1:5 dilute) loaded in each well and incubation was carried out for 15 min at room temperature.
4) Absorbance was read in microplate reader equipped with 600 nm filter.
5) Concentration of protein from haemolymph of normal and virosed larvae was determined from standard curve (Plate 4). Each sample was measured in triplicate to minimize the error.

3.2.4 Determination of enzyme level in different age group

Different instar larvae of *H. armigera* i.e. 3rd instar as a susceptible and 5th instar as resistant were used to determine the level of Phenoloxidase in haemocytes and plasma of haemolymph of *H. armigera*. The larvae were treated with predetermined HaNPV concentration as per LC_{50} values for 3rd and 5th instar as said earlier.

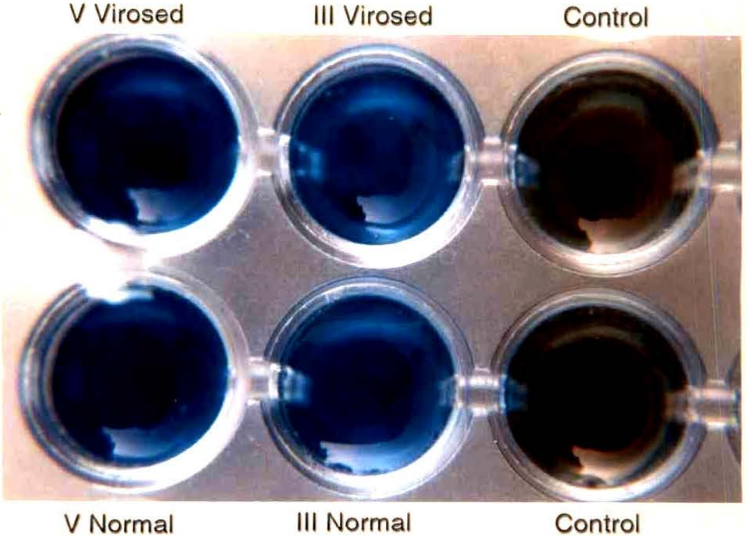
Phenoloxidase activity was determined by using the methodology of Anderson *et al.*, (1989). Two hundred µl of haemolymph was withdrawn from the larvae after 0, 24, 48 and 72 hrs of treatment by puncturing at the base of 2nd proleg. The haemolymph so withdrawn was collected into 300 µl of neutralized $(NH_4)_2SO_4$, to achieve 40 per cent saturation. The mixture was kept undisturbed for 30 min. A portion of this mixture was retained as whole haemolymph 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 absorbance at 490 nm was measured at every minute interval on UV spectrophotometer and specific PO activity was determined and expressed in Units/mg protein/min as per Hung and Boucles (1996) and Anderson *et al.*, (1989).

3.2.5 Characterization of haemolymph phenoloxidase

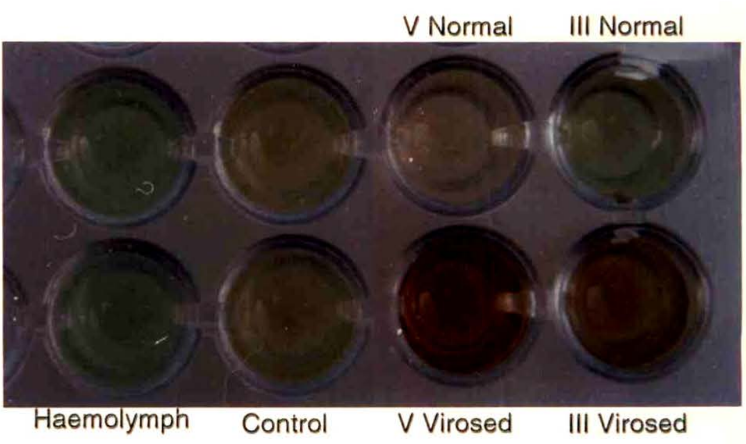
The haemolymph phenoloxidase enzyme from normal and virosed larvae of *H. armigera* were characterized by studying some of the biochemical parameters. Microplate assay was exploited in all the characterization experiments as described by Lockey and Ourth (1992) with slight modification (Plate 2). All the samples were measured in triplicate.

3.2.5.1 Km and Vmax

Michaelis constant (Km) and Maximal velocity (Vmax) were worked out for the haemolymph phenoloxidase enzyme from normal and virosed larvae of *H. armigera* by using different substrates viz., L-3-4 Dihydroxy phenyl alanine (L- DOPA), DL-3-4-Dihydroxy phenyl alanine (DL-DOPA), L-Tyrosine, Dopamine Hydrochloride 3-hydroyxtyramine chloride (Dopamine), 4-Methylbrenzcatecholin. Different molar concentrations of all the above substrates viz., 15, 7.5, 5, 1.5 and 0.15 µM were used for



a. Protein estimation of normal and virosed III, V instar larvae of *H. armigera*.



b. Phenoloxidase from normal and virosed III, V instar larvae of *H. armigera*

Plate 2. Microplate Assay of Haemolymph Protein and Phenoloxidase

this study. For calculating Michaelis constant, 1/S values and 1/V values for corresponding absorbance were calculated.

1) Fifty microliter of haemolymph from normal and virosed larvae of *H. armigera* was taken in the wells of microplate.
2) Volume was made upto 150µl by using 0.01M phosphate buffer (pH 7.0).
3) 50 µl of substrate solution [Different concentration of all above substrates viz., 7.5, 1.5, 0.15, 0.015 and 0.0015 µM in distilled water] was loaded in each well.
4) The plate was incubated in dark at room temperature for 10 min and was read in microplate reader equipped with a 490 nM filter.
5) The specific activity of Phenoloxidase was expressed as unit of activity per mg of protein per min. One unit of activity was defined as the amount of enzyme that increases the absorbtion by 0.001 units per min (Anderson *et al.*, 1989).
6) Michaelis - Mentone Equation/graph and Double Reciprocal Plot was drawn for determining the Km and Vmax values for normal and virosed larvae against each substrate as given above

3.2.5.2 Substrate specificity

Substrate specificity was determined by studying haemolymph phenoloxidase from normal and virosed larvae of *H. armigera* with five different substrates viz., L-3-4 Dihydroxy phenyl alanine (L- DOPA), DL-3-4- Dihydroxy phenyl alanine (DL-DOPA), L-Tyrosine, Dopamine Hydrochloride 3-hydroytyramine chloride (Dopamine), 4-Methylbrenzcatecholin concentration of substrate. Same protocol of microplate assay was followed.

Enzyme activity was determined by performing the microplate assay with 50 µl of haemolymph obtained from normal and virosed larvae of *H. armigera.*

3.2.5.3 Optimum temperature

1) Fifty microliter of haemolymph from normal and virosed larvae of *H. armigera* was taken in the 500µl of PCR tube
2) Volume was made up upto150µl by using 100µl 0.01M phosphate buffer (pH 7.0).
3) Followed by 15 min. incubation at different temperatures viz 10,20,30,40,50,60,70 and 80 °C. in thermal cycler (Applied Biosystems 9700)

4) This 150 µl of solution was transferred to microplate just after the incubation, 50 µl of substrate solution (0.0040g Dopamine1.0 ml distilled water) was added to the plate and was incubated in dark at room temperature for 10 min and was read in Microplate reader equipped with a 490 nM filter.

5) A line graph was plotted to determine the optimum temperature range for phenoloxidase.

3.2.5.4 Thermal stability

The thermal stability of the haemolymph phenoloxidase enzyme from normal and virosed larvae of *H. armigera* was studied by incubating the haemolymph at different temperatures viz., 10,20,30,40,50,60,70 and 80 °C for different time intervals ranging from 10 to 80 min followed by rapid cooling to 0°C, the incubation was carried out in thermal cycler. The enzyme activity was measured by the microplate assay as described above.

3.2.5.5 Optimum pH

1) Fifty microliter of haemolymph from normal and virosed larvae of *H. armigera* was taken and pre incubated for 30 min with 100 µl 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 haemolymph absorbance.
2) 50 µl of substrate solution (0.0040g Dopamine in 1.0 ml distilled water) was added to the plate and incubated in dark at room temperature for 10 min.
3) The plate was read in microplate reader equipped with a 490 nM filter.
4) A line graph was plotted to determine the optimum pH range for the activity of haemolymph phenoloxidase

3.2.5.6 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.

3.2.6 Determination of HaNPV toxicity and phenoloxidase levels from different colour *H. armigera* larvae

Third and fourth instar larvae of *H. armigera* of different colour were collected from the field and were exposed to the different concentration of HaNPV. LC_{50} values were worked out as determined previously. Similarly, phenoloxidase level in different colour larvae infected with HaNPV was worked out as stated earlier and the co-relation if any in between them was studied.

3.2.7 Study of *in vitro* inhibition of phenoloxidase

Using different PO inhibitors, inhibition studies were carried out. Ethylenediamine tetra acetic acid (EDTA), Ethylene glycol-bis (β-Aminoethyl Ether) N, N, N, N-tetraacetic acid (EGTA), Soybean trypsin inhibitor (STI) (Hung and Boucias, 1996), Phenylthiourea (PTU) (Barrett and Andersen, 1981), Phenyl methyl sulfonyl fluoride (PMSF) (Aspan *et al.*, 1990), Sodium dodecyl sulfate (SDS) (Lockey and Ourth, 1992), Dimethyl sulfoxide (DMSO) and Garlic extract (Mantawy and Mahmoud, 2002) were used at different concentrations.

In this inhibition study, all the following inhibitors were used in 2x concentration as EDTA (25 mM, 10 mM, 2.5 mM), EGTA (25 mM, 10 mM, 5 mM), STI (10 mg, 1mg, 0.1mg), PTU (25 mM, 10 mM, 5 mM), PMSF (25 mM, 10 mM, 2.5 mM), SDS (1%, 0.1%, 0.05%), DMSO (10%, 1%, 0.1%), and Garlic extract (10%, 5%, 1%).

As given with each inhibitor individually 25 µl of different concentrations of all above said inhibitors were mixed with 25µl of haemolymph from normal and virosed larvae of *H. armigera* in each well followed by 30 min incubation at room temperature. Phosphate buffer (pH 7.0) 100µl 0.01M was added in each well. Afterward 50 µl of substrate solution (0.0040g Dopamine1.0 ml distilled water) 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 25µl of haemolymph plus 25µl distilled water instead of inhibitor mixture; reduction in the intensity of colour developed due to addition of inhibitors was calculated.

3.2.8 Effect of different phenoloxidase inhibitors in combination with HaNPV

From the above inhibitor study, the inhibitor that found effective in inhibiting the phenoloxidase activity *in vitro* were taken in different concentration and incorporated in the semi-synthetic diet with or without HaNPV and resultant mortality were compared.

3.2.9 Electrophoresis study

In this study, we attempted to explore the question i.e. Which difference exist in the haemolymph phenoloxidase isozymes from normal and virosed larvae of *H. armigera*?

Nondenaturing 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 haemolymph was loaded in the gel and electrophoresis was conducted (Bidochka *et al.*, (1989) at a constant current of 35mA (Hoefer PS-500 power unit) for 7-8 hr at 4°C.

After electrophoresis, non specific phenoloxidase band in the gels were revealed by staining them with L-DOPA. Gel was placed 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. The staining solution was replaced by distilled water.

3.2.10 Total haemocyte count

To study the changes in haemocytes count of different age larvae of *H. armigera,* 3rd and 5th instar larvae were grouped and each group was divided into two sub groups of normal and NPV treated larvae. The test larvae were starved for 6 hr and fed on surface contaminated diet piece of 1 cm^3 treated with 0.2 ml of different concentration of HaNPV as the pre determined LC_{50} values whereas normal larvae were fed on diet treated with distilled water and allowed to feed for 24h, after which they were transferred to fresh diet. Three replications of 10 larvae were maintained. Fresh haemolymph was drawn from the larvae of each subgroup everyday till mortality or pupation THC was done according to the method described by Kalia *et al.*, (2001) with slight modification. The fresh haemolymph was collected by puncturing 2nd proleg, 20μl haemolymph was diluted in 180 μl of anticoagulant

buffer (0.01 M EDTA, 0.2 M Glucose, 0.062 M Sodium chloride, 0.03 M Trisodium citrate, and 0.026 M citric acid). A drop of diluted haemolymph was put to fill the hemocytometer and covered with cover slip. Care was taken to avoid air bubble. The haemocytes were counted from five 1-mm size square (the four corner and one central square) of the Neubar Hemocytometer. The total haemocyte count was calculated by multiplying average count per square of 1mm with a factor of 10 (depth of chamber is 0.1mm which was multiplied by 10 to get depth of 1mm) and with a dilution factor. The THC is a mean of 10 replications. When the variation in cell count between any two 1-mm squares was greater than 10%, the entire count was discarded and a new count was made. If clumping of the cells was observed, the count was also discarded (Shpario, 1979).

CHAPTER IV

EXPERIMENTAL FINDINGS

Studies on phenoloxidase activity and melanization in *Helicoverpa armigera* (Hubner) exposed to HaNPV were carried out in laboratory of Department of Entomology and Insect Biotechnology laboratory of Dr. PDKV, Akola during 2003 and 2004 with the objectives to study age related resistance in *H. armigera*, associated role of phenoloxidase (PO), characterization of haemolymph PO and Total haemocyte count in normal and virosed larvae. The experimental findings are described here with.

4.1 Age related bioassay of *H. armigera* with HaNPV

The data on LD_{50} values for second, third, fourth and fifth instar larvae of *H. armigera* presented in Table 4 and Fig.1 revealed that the critical dose for affecting 50 per cent mortality i.e. LD_{50} in second instar larvae was observed as 2.1×10^3 POB/ml which was lowest when compared with the LD_{50} value for third instar of 1.0×10^4 POB/ml and 3.4×10^5 and 2×10^6 POB/ml, respectively for fourth and fifth instar larvae of *H. armigera,* these LD_{50} values were quite higher as compared to that of second and third instars.

The fiducial limit observed were $0.9 \times 10^3 - 4.8 \times 10^3$ POB/ml with slope 0.72 for second instar and was $4.4 \times 10^3 - 2.2 \times 10^4$ with the slope of 0.60 for third instar whereas $1.6 \times 10^5 - 6.9 \times 10^5$ and $7.9 \times 10^5 - 5.1 \times 10^6$ POB/ ml with slope of 0.55 and 0.47,respectively, for fourth and fifth instar larvae of *H.armigera.*

The slope value for second instar was significantly more i.e. 0.72 followed by the third instar indicating sensitivity of the doses in second and third instars. However, the slopes of fifth instar significantly differed from second and third instar, although the slope of latter was in parity indicating that from third instar onwards, the LD_{50} values increase very much significantly.

Based on the comparison on LD_{50} values amongst different instars (fig 1), the second instar larvae were found to be the most susceptible i.e. 4.91, 161.73, 960.57 time more susceptible as compared to third, fourth and fifth instar larvae of *H. armigera,* respectively. The susceptibility decreased in third instar, as third instar larvae were 32.97 and 195.83 time

Table 4: LD_{50} of NPV to *H. armigera* larvae

Sr. No	Instar	LD_{50} (POB/ml)	Fiducial limit (POB/ml)		Chi Square	Slope (b)	Susceptibility over instar (Folds)		
			Lower	Upper			III	IV	V
1	Second	2.1×10^3	0.9×10^3	4.8×10^3	0.50	0.72±0.13	4.91	161.73	960.57
2	Third	1.0×10^4	4.4×10^3	2.2×10^4	0.67	0.60±0.15	-	32.97	195.83
3	Fourth	3.4×10^5	1.6×10^5	6.9×10^5	1.90	0.55±0.08	-	-	15.9
4	Fifth	2.0×10^6	7.9×10^5	5.1×10^6	2.04	0.47±0.11	-	-	-

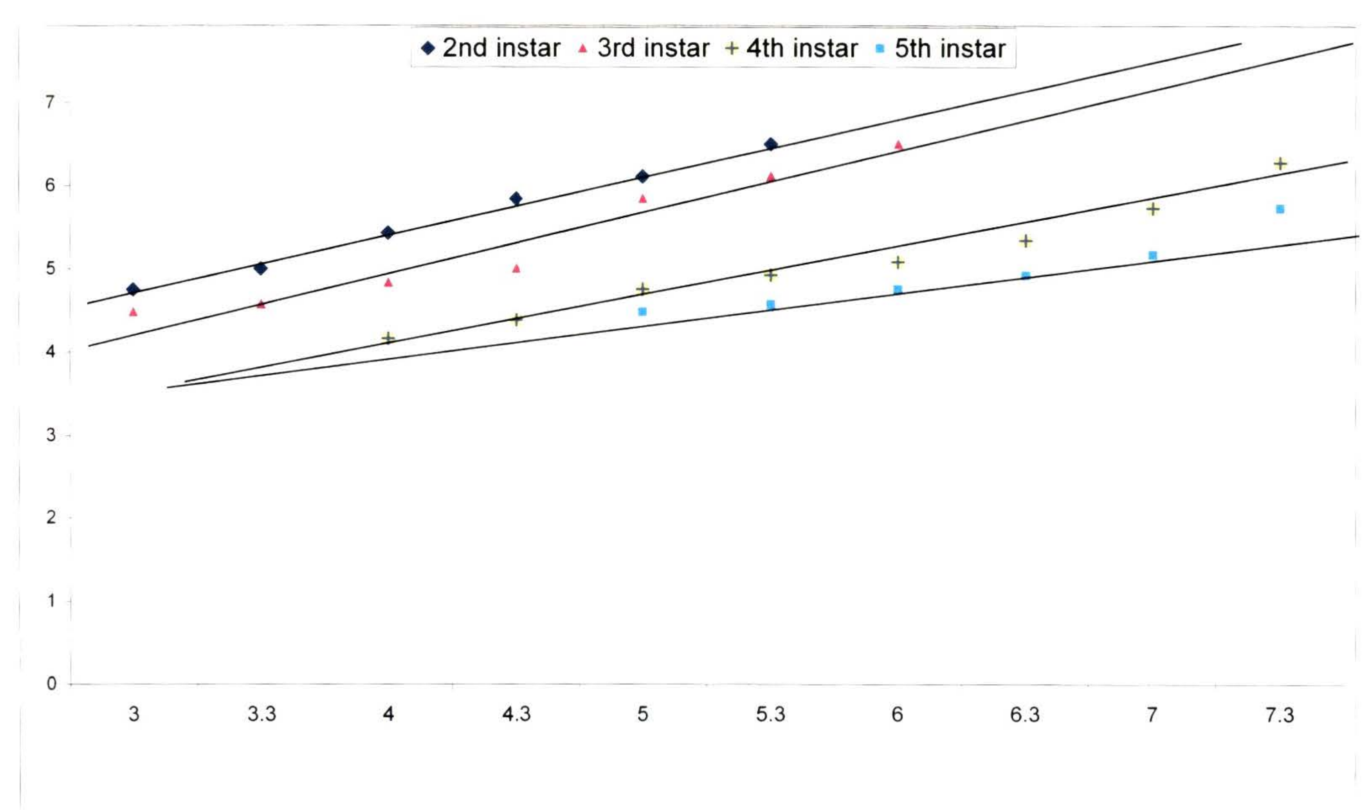


Fig.1: Probit Line of different instars of *H.armigera* larvae

more susceptible than fourth and fifth instar larvae further decrease in susceptibility was observed in fourth instar, as it was just 15.9 time more than fifth instar larvae. Thus, these results indicated that the second instar larvae were most susceptible even at lowest dose of HaNPV (2.1 x 10^3 POB/ml), while the fifth instar was least susceptible and required very high dose indicating its résistance with higher LD_{50} value of 2.0 x 10^6 POB/ml. The susceptibility decreased with the advancement in the age of larva.

4.2 Determination of phenoloxidase in different age group of *H. armigera*

So as to ascertain the relationship between the age and susceptibility/resistance of *H. armigera* to HaNPV, the determination of phenoloxidase in cellular and humeral fraction of III instar and V instar of *H. armigera* was done by microplate assay and the results are presented in Table 5, 6 and depicted in Fig. 2, 3. The quantitative assay for phenoloxidase activity as Unit/mg protein/min, indicated that there was increased PO enzyme activity in both cellular and plasma fraction of the haemolymph in virosed larvae as compared to normal. However, it was not so in the cellular fraction of 5th instar larvae where the activity was oscillating.

In case of the normal 3rd instar larvae, the PO activity in cellular fraction initially at 0 hr was found to be 322.99 Unit/mg protein/min. and it increased steadily up to 72 hr and reached to 411.32 Unit/mg protein/min However, in case of plasma fraction at 0 hr, the PO activity was not detected in normal larvae up to 48 hr but indicated the change at 72 hr and PO activity was found as 152.63 Unit/mg protein/min.

In case of virosed larvae for the 3rd instar, the cellular fraction showed the PO activity of 325.41 Unit/mg protein/min initially at 0 hr, one-way ANOVA analysis showed insignificant difference as compared to normal at 0.05 %, which subsequently increased and reached to

Table 5: Phenoloxidase activity in cellular and plasma fraction of normal and virosed 3rd instar larvae of *H.armigera*

Post Infection Period	Normal (Unit/mg protein/min)		Virosed (Unit/mg protein/min)		Per cent increase	
	Cellular	Plasma	Cellular	Plasma	Cellular	Plasma
0hr.	322.99±3.3	N.D*	325.41±4.9	N.D	01	N.D
24hr	346.02±2.6	N.D	680.17±4.9	21.62±2.14	96	N.D
48hr	361.49±4.7	N.D	807.37±3.0	168.6±4.07	123	N.D
72hr	411.32±3.5	152.63±3.3	902.24±3.7	272.2±3.67	119	78

* N.D- Not Detected

902.24 Unit/mg protein/min after 72 hr. One-way ANOVA analysis showed significant difference in PO activity as compared to normal. Whereas in the plasma fraction of the same, the PO activity was not detected initially at 0 hr, however, after 24 hr, it was found to be 21.62 Unit/mg protein/min and increasing trend continued up to 72 hr (272.20 Unit/mg protein/min) which was significantly more than normal larvae on the basis of one-way ANOVA at 0.05 %.

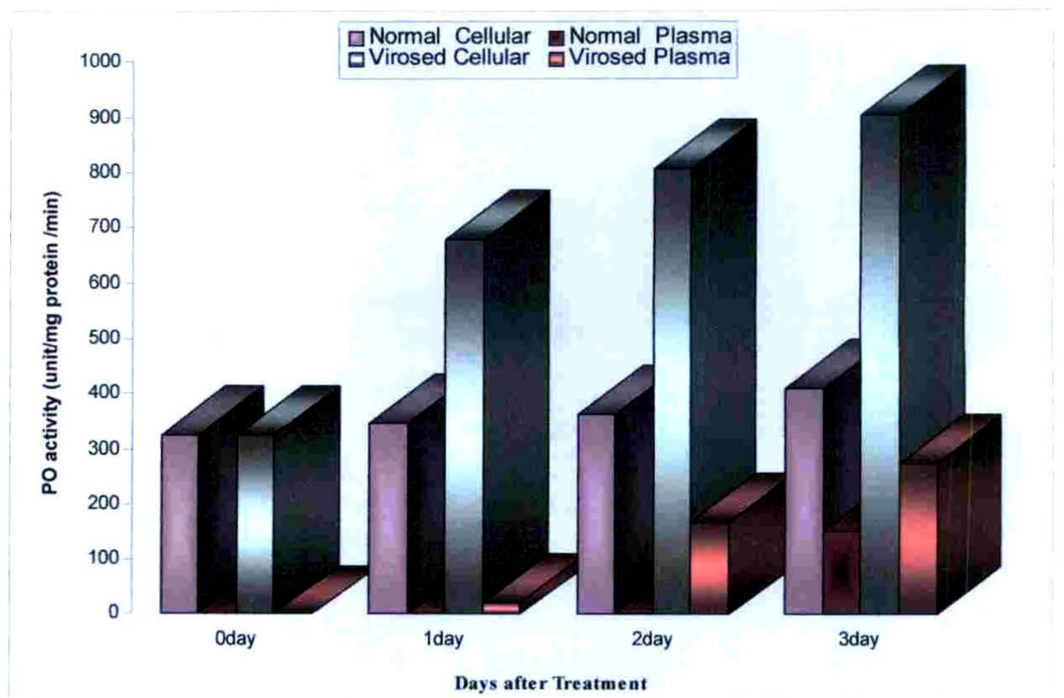


Fig.2: Phenoloxidase activity in cellular and plasma fraction of normal and virosed 3rd instar larvae of *H.armigera*

The fold increase in PO activity in virosed as compared to normal larvae was also worked out. It was found that in the cellular fraction of 3rd instar at 0 hr there was negligible increase in PO activity. However, later on at 24 hr, it increased by 96 per cent and subsequently an increase of 123 and 119 per cent was observed, respectively at 48 and 72 hr after treatment. Whereas, in case of plasma fraction at 0 hr, there was no change and continued up to 48 hr. However, the PO activity was increased by 78 per cent at 72 hr after treatment (Table 5).

In normal 5th instar larvae of *H. armigera* the PO activity in cellular fraction was found to be 427.04 Unit/mg protein/min and it increased up to 506.70 Unit/mg protein/min at 48 hr.

But at 72 hr, reduction of PO activity was observed and it recorded 431.49 Unit/mg protein/min. In plasma fraction of same group, PO activity at 0 hr was found to be 229.18 Unit/mg protein/min and later on increased up to 392.04 Unit/mg protein/min at 72 hr (Table 6).

Table 6: Phenoloxidase activity in cellular and plasma fraction of normal and virosed 5th instar larvae of *H.armigera*

Post Infection Period	Normal (Unit/mg protein/min)		Virosed (Unit/mg protein/min)		Per cent increase	
	Cellular	Plasma	Cellular	Plasma	Cellular	Plasma
0hr	427.04±2.7	229.18±3.8	645.71 ±4.6	234.32 ±5.7	34	2
24hr	486.18±2.5	277.93±3.9	621.43 ±4.9	319.74 ±5.3	27	15
48hr	506.7 ±3.2	391.33±43	889.89 ±4.6	732.77 ±6.1	75	87
72hr	431.49±2.0	392.04±4.1	508.99 ±4.1	968.34 ±6.1	17	147

Cellular fraction of virosed 5th instar larvae of *H. armigera* had a significantly higher PO enzyme level at 0 hr i.e. 645.71 Unit/mg protein/min as compared to normal on the basis of one-way ANOVA at 0.05 %, which later decreased to 621.43 Unit/mg protein/min which was also significant as compared to normal. Afterwards it subsequently increased up to 889.89 Unit/mg protein/min at 48 hr. However the oscillatory reaction was observed again at 72 hr and reduction in PO activity up to 508.99 Unit/mg protein/min was observed, both of these values were significantly more than their normal counterpart on the basis of one-way ANOVA at 0.05 %.

However, in case of plasma fraction of virosed 5th instar larvae at 0 hr, the enzyme activity (234.32 Unit/mg protein/min) did not differ significantly as compared to that of normal (229.18 Unit/mg protein/min) on the basis of one-way ANOVA at 0.05 %. Titer of PO activity was found to increase as the time after reaction of larvae increased up to at 72 hr and it reached to 968.34 Unit/mg protein/min which was significantly more than that of normal larvae (434.49 Unit/mg protein/min) on the basis of one-way ANOVA at 0.05 %.

The percent increase in PO activity in virosed as compared to normal larvae was also worked out. It was observed that in 5th instar cellular fraction immediately after treatment, there was 34 per cent increase in PO activity, later on at 24 hr it increased by 27 per cent and subsequently increased by 75 per cent at 48 hr. However, the increase was just by 17 per cent at 72 hr. Whereas in case of plasma fraction at 0 hr, there was increase of just 2 per cent, at 24

hr, 15 per cent increase was noted, whereas, at 48 hr and 72 hr after treatment there was change of 87 and 147 per cent in PO activity (Table 6).

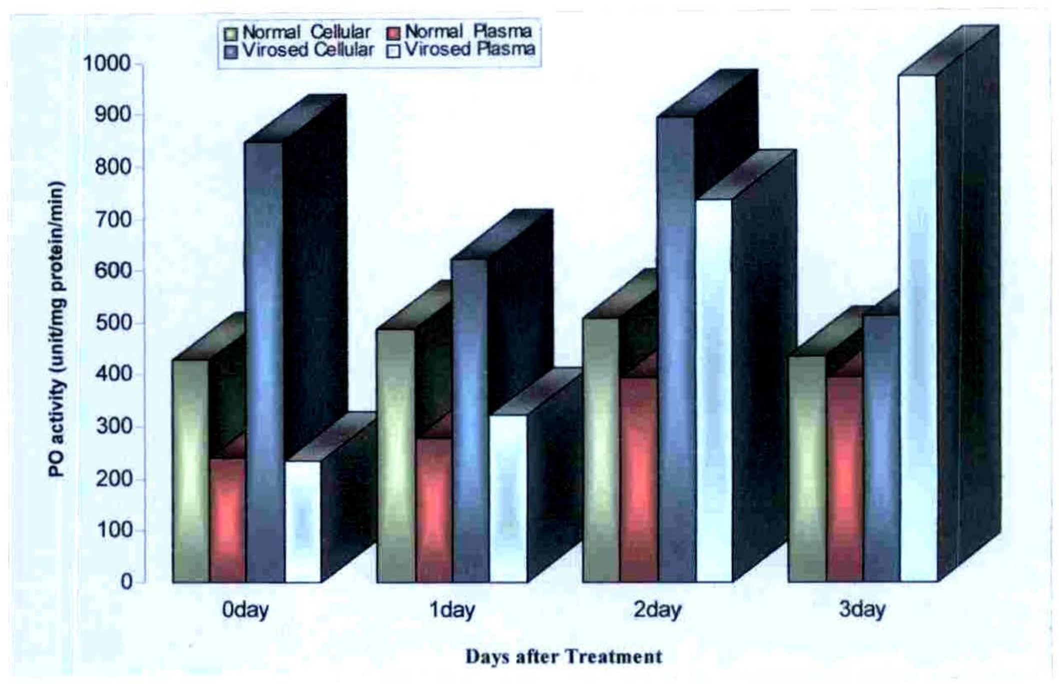


Fig. 3: Phenoloxidase activity in cellular and plasma fraction of normal and virosed 5th instar larvae of *H.armigera*

So in general the PO activity in plasma fraction of virosed larvae increased considerably after 48 hr when compared with normal larvae of *H. armigera*, however in case of cellular fraction, PO activity was found to increase in 3rd instar of virosed larvae of *H.armigera*. In 5th instar, the PO activity in cellular fraction of virosed larvae was found to decrease as the age increased, the quantity of PO in 5th instar larvae was more as compared to 3rd instar larvae of *H. armigera*. Percent increase in PO activity was also more in 5th instar as compared to 3rd instar larvae of *H. armigera*.

4.3 Characterization of phenoloxidase from normal and virosed larvae of *H. armigera*

4.3.1 Kinetics of phenoloxidase

The kinetics of phenoloxidase as affected due to HaNPV was studied by microplate assay using haemolymph with different substrate viz., L-Tyrosine, L-DOPA, Methylcatechol, Dopamine and DL-DOPA. These substrates were tested in virosed and normal larvae of

H.armigera and the phenoloxidase (PO) activity was measured. Lineweaver -Burk double reciprocal plot was drawn in between concentration of substrate and absorbance and Michaelis constant (Km) and maximum velocity (Vmax) was deduced for phenoloxidase from normal and virosed larvae of *H. armigera.*

4.3.1.1 L- Tyrosine

The kinetics of PO was studied by taking various concentration of L-Tyrosine viz., 15, 7.5, 5, 1.5, 0.15 mM. Normal larvae of *H. armigera* showed activity of 0.30854, 0.290909, 0.266667, 0.264463 and 0.257851 ΔA/mg/min, respectively against the substrate values. Virosed larvae of *H. armigera* showed PO activity of 0.351601, 0.324588, 0.306297, 0.264463 and 0.257851 ΔA/mg/min corresponding to above substrate concentrations, respectively. Lineweaver-Burk reciprocal equation was plotted by conspiring substrate concentration [1/S] on X- axis and PO absorbance [1/V] on Y-axis [Table 7, Fig. 4 and 5].

Table 7: Kinetics of haemolymph phenoloxidase from virosed and normal V instar larvae of *H. armigera* (substrate L-tyrosine)

Sr. No.	(S) mM.	Normal			Virosed		
		PO [V] ΔA/mg/min	1/(S)	1/(V)	PO[V] ΔA/mg/min	1/(S)	1/(V)
1	15	0.30854	0.066667	3.241071	0.351601	0.066667	2.829339
2	7.5	0.290909	0.133333	3.4375	0.324588	0.133333	3.046581
3	5	0.266667	0.2	3.75	0.306297	0.2	3.255204
4	1.5	0.264463	0.666667	3.78125	0.264463	0.666667	3.78125
5	0.15	0.257851	6.66667	3.878205	0.257851	6.66667	3.878205

The Km of PO for the substrate was determined from the intercept on the X-axis. Like wise Vmax was determined from the intercept on Y-axis. Accordingly the Km values of PO for L-Tyrosine was found to be 1.36 and 1.21 mM in normal and virosed population, respectively, showed 12.39 per cent decrease in Km (Table 12). Similarly, Vmax values were 0.333 and 0.377 ΔA/mg/min in normal and virosed population respectively, showed 13.21 per cent increases as compared to normal strain. Thus, it was found that there was increase in velocity of enzyme (increase in Vmax) and increase in affinity of substrate towards PO (decrease in Km).

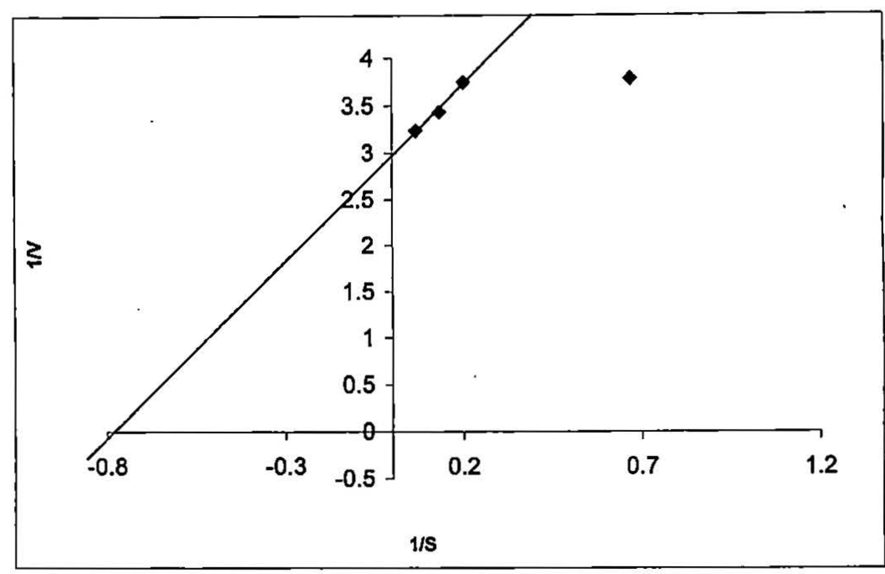


Fig. 4: Lineweaver-Burk plot for PO activity of normal V instar larvae of *H. armigera* by using L-tyrosine as substrate

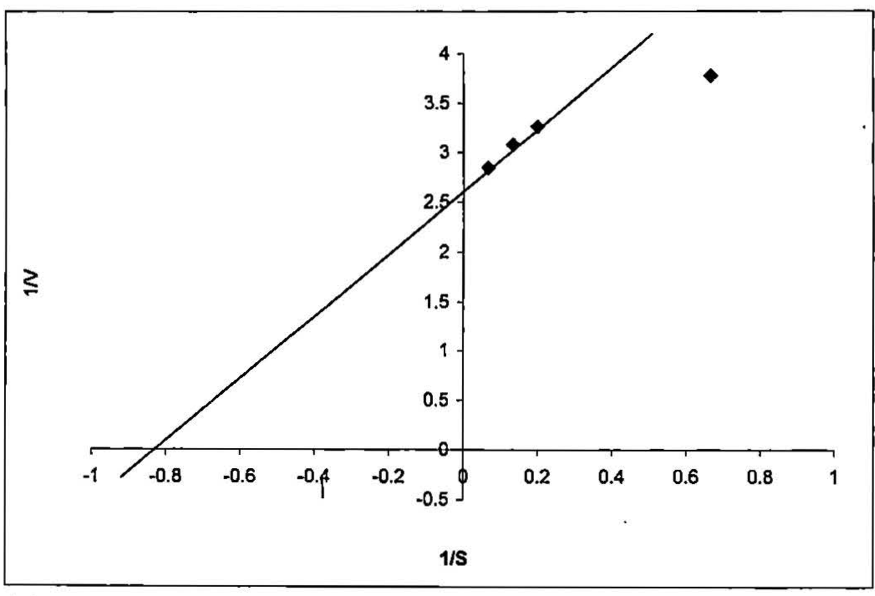


Fig. 5: Lineweaver-Burk plot for PO activity of virosed V instar larvae of *H. armigera* by using L-tyrosine as substrate

4.3.1.2 L-Dopa

The kinetics of PO was studied by taking various concentration of L-DOPA viz., 15, 7.5, 5, 1.5, 0.15 mM. Normal larvae of *H. armigera* showed PO activity of 1.765745, 1.840133, 2.32465, 3.267323 and 3.655947 ΔA/mg/min, respectively. Virosed larvae of *H. armigera* showed PO activity of 1.02081, 1.906513, 2.338918, 2.890127 and 3.107877 ΔA/mg/min, corresponding to above substrate concentrations. respectively. Lineweaver-Burk reciprocal equation was plotted by conspiring [1/S] on X- axis and [1/V] on Y-axis [Table 8, Fig. 6 & 7].

Table 8: Kinetics of haemolymph phenoloxidase from virosed and normal V instar larvae of *H. armigera* (substrate L-dopa)

Sr. No.	(S) mM.	Normal			Virosed		
		PO [V] ΔA/mg/min	1/S	1/V	PO [V] ΔA/mg/min	1/S	1/V
1	15	0.566333	0.066667	1.765745	0.979614	0.066667	1.02081
2	7.5	0.543439	0.133333	1.840133	0.524518	0.133333	1.906513
3	5	0.430172	0.2	2.32465	0.427548	0.2	2.338918
4	1.5	0.306061	0.666667	3.267323	0.346006	0.666667	2.890127
5	0.15	0.273527	6.66667	3.655947	0.321763	6.66667	3.107877

The Km values of PO for L-DOPA were found to be 2.85 and 1.25 mM in Normal and virosed population, respectively, showed 128 per cent decrease in Km (Table 12). Similarly, Vmax values were 0.667 and 0.832 ΔA/mg/min in normal and virosed population, respectively, showed 24.73 per cent increase as compared to Normal strain. Thus, it was found that there was increase in velocity of enzyme (increase in Vmax) and increase in affinity of substrate towards PO (decrease in Km).

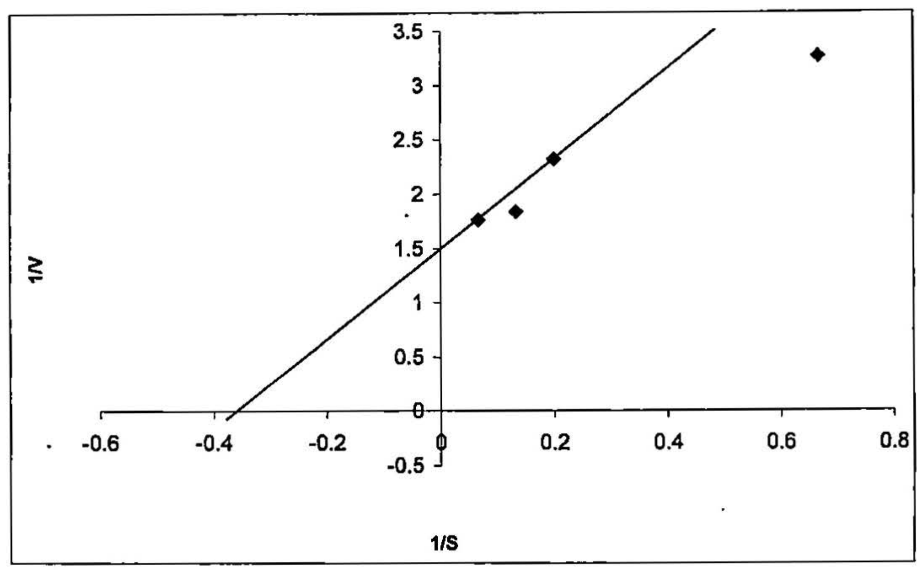


Fig. 6: Lineweaver-Burk plot for PO activity of normal V instar larvae of *H. armigera* by using L-Dopa as substrate

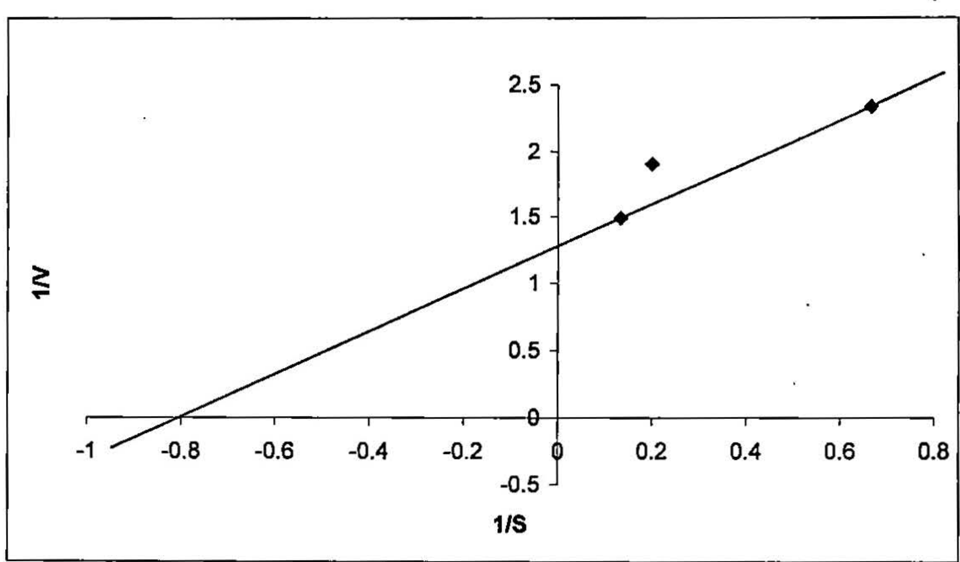


Fig. 7: Lineweaver-Burk plot for PO activity of virosed V instar larvae of *H. armigera* by using L-Dopa as substrate

4.3.1.3 Methylcatechol

The kinetics of PO were studied by taking various concentration of Methylcatechol viz., 15, 7.5, 5, 1.5, 0.15 mM. Normal larvae of *H. armigera* showed activity of 3.359919, 3.373577, 8.468367, 12.02754 and 92.21111, ΔA/mg/min, respectively. Virosed larvae of *H. armigera* showed PO activity of 1.70904, 1.848269, 2.003311, 2.844828 and 2.853774 ΔA/mg/min, corresponding to above substrate concentrations. Lineweaver -Burk reciprocal equation was plotted by conspiring [1/S] on X- axis and [1/V] on Y-axis [Table 9, Fig. 8 & 9].

Table 9: Kinetics of haemolymph phenoloxidase from virosed and normal V instar larvae of *H.armigera* (substrate Methylcatechol)

Sr. No.	(S) mM.	Normal			Virosed		
		PO [V] ΔA/mg/min	1/S	1/V	PO [V] ΔA/mg/min	1/S	1/V
1	15	0.297626	0.066667	3.359919	0.585124	0.066667	1.70904
2	7.5	0.296421	0.133333	3.373577	0.541047	0.133333	1.848269
3	5	0.118087	0.2	8.468367	0.499174	0.2	2.003311
4	1.5	0.083143	0.666667	12.02754	0.351515	0.666667	2.844828
5	0.15	0.010845	6.66667	92.21111	0.350413	6.66667	2.853774

The Km values of PO for Methylcatechol were found to be 6.67 and 1.66 mM in normal and virosed population, respectively, showed 225.60 per cent decrease in Km (Table 12). Similarly, Vmax values were 0.454 and 0.667 ΔA/mg/min in normal and virosed population respectively, showed 46.91 per cent increase as compared to normal strain. Thus, it was found that there was increase in velocity of enzyme (increase in Vmax) and increase in affinity of substrate towards PO (decrease in Km).

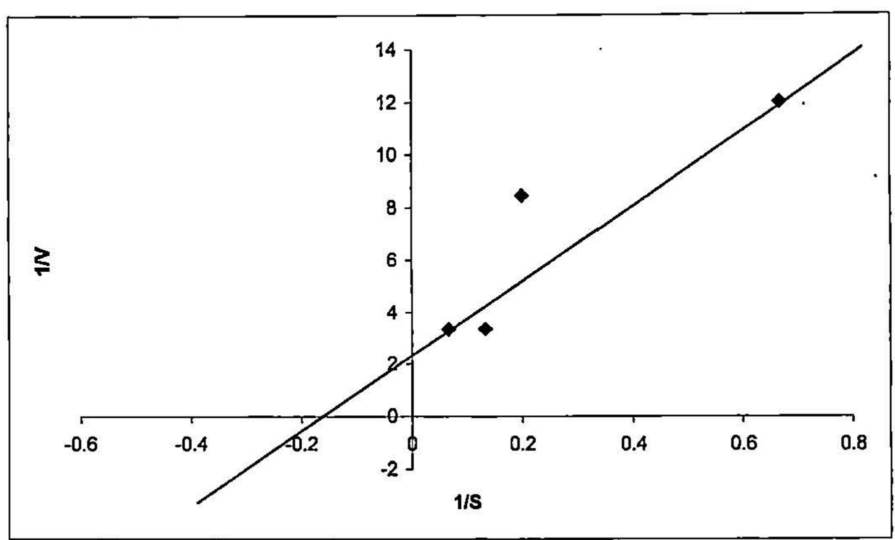


Fig. 8: Lineweaver-Burk plot for PO activity of normal V instar larvae of *H. armigera* by using Methylcatechol as substrate

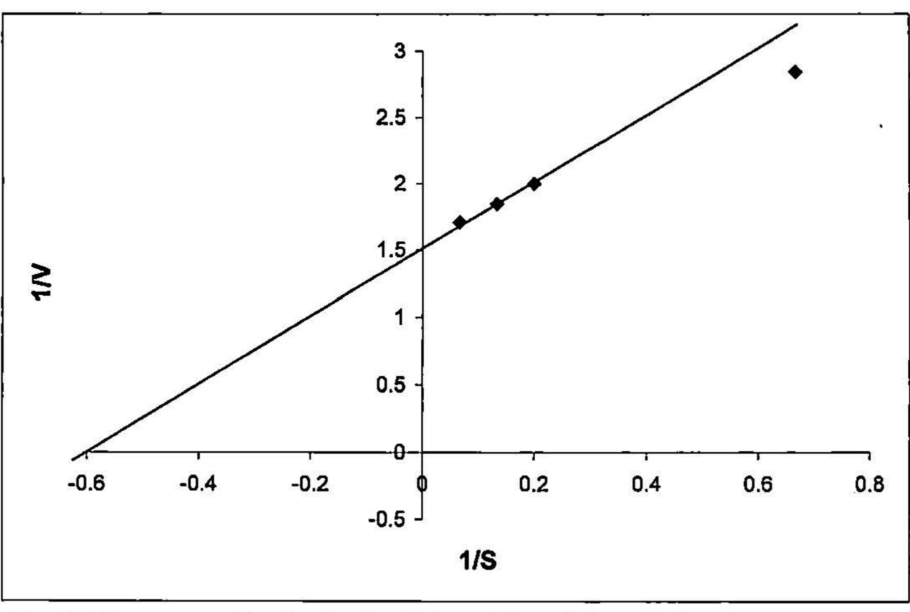


Fig. 9: Lineweaver-Burk plot for PO activity of virosed V instar larvae of *H. armigera* by using Methylcatechol as substrate

4.3.1.4 Dopamine

The kinetics of PO were studied by taking various concentration of Dopamine viz., 15, 7.5, 5, 1.5, 0.15 mM Normal larvae of *H. armigera* showed activity of 1.522752, 2.609748, 5.723448, 6.802459 and 7.279825 ΔA/mg/min, respectively. Virosed larvae of *H. armigera* showed PO activity of 0.987486, 1.024266, 3.229537, 3.63 and 3.76556 ΔA/mg/min, corresponding to above substrate concentrations, respectively. Lineweaver-Burk reciprocal equation was plotted by conspiring [1/S] on X- axis and [1/V] on Y-axis [Table 10, Fig. 10 and 11].

Table 10: Kinetics of phenoloxidase from virosed and normal haemolymph of V instar larvae of *H. armigera* (substrate Dopamine)

Sr. No.	(S) mM.	Normal			Virosed		
		PO [V] ΔA/mg/min	1/S	1/V	PO [V] ΔA/mg/min	1/S	1/V
1	15	0.656706	0.066667	1.522752	1.012672	0.066667	0.987486
2	7.5	0.383179	0.133333	2.609748	0.976309	0.133333	1.024266
3	5	0.17472	0.2	5.723448	0.309642	0.2	3.229537
4	1.5	0.147006	0.666667	6.802459	0.275482	0.666667	3.63
5	0.15	0.137366	6.66667	7.279825	0.265565	6.66667	3.76556

The Km values of PO for dopamine was found to be 6.25 and 5.55μM in normal and virosed population, respectively, showed 12.61 per cent decrease in Km (Table 12). Similarly, Vmax values were 1.053 and 1.587 μM mg/protein/min in normal and virosed population respectively, showed 50.71 per cent increase as compared to normal strain. Thus, it was found that there was increase in velocity of enzyme (increase in Vmax) and increase in affinity of substrate towards PO (decrease in Km).

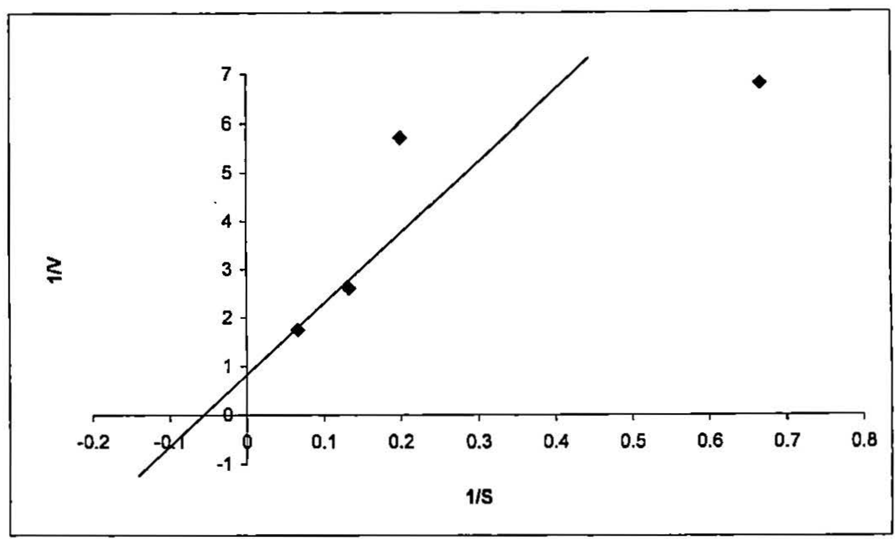


Fig. 10: Lineweaver -Burk plot for PO activity of normal V instar larvae of *H. armigera* by using Dopamine as substrate

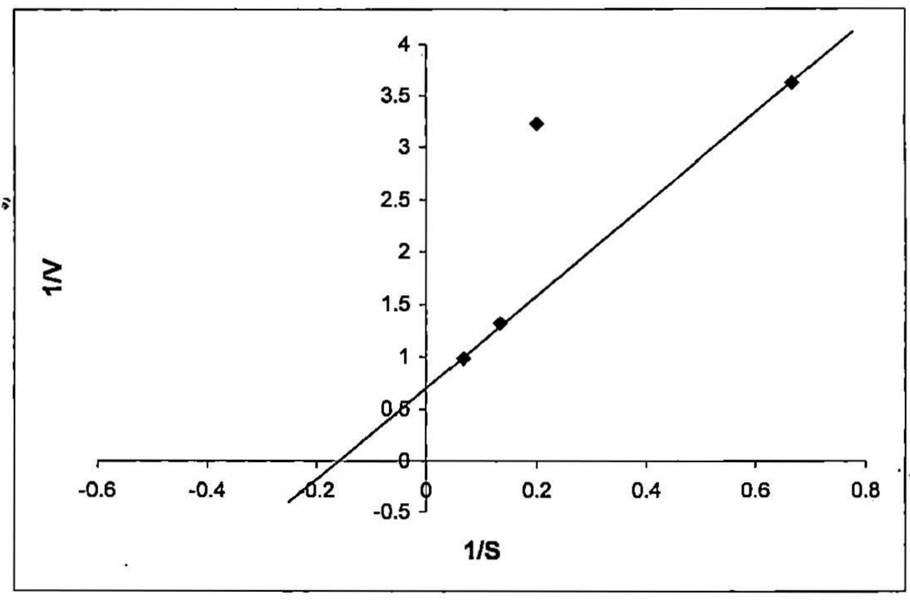


Fig. 11: Lineweaver-Burk plot for PO activity of virosed V instar larvae of *H. armigera* by using Dopamine as substrate

4.3.1.5 DL-Dopa

The kinetics of PO were studied by taking various concentration of DL-Dopa viz., 15, 7.5, 5, 1.5, 0.15 mM. Normal larvae of *H. armigera* showed activity of 2.059305, 2.144444, 2.784899, 2.85189 and 3.267323 ΔA/mg/min, respectively. Virosed larvae of *H. armigera* showed PO activity of 1.263928, 1.417969,2.034753, 2.26875 and 3.928571 ΔA/mg/min, corresponding to above substrate concentrations, respectively. Lineweaver-Burk reciprocal equation was plotted by conspiring [1/S] on X- axis and [1/V] on Y-axis [Table 11, Fig. 12 and 13].

Table 11: Kinetics of phenoloxidase from virosed and normal haemolymph of V instar larvae of *H. armigera* (substrate DL-Dopa)

Sr. No.	(S) mM.	Normal			Virosed		
		PO [V] ΔA/mg/min	1/S	1/V	PO [V] ΔA/mg/min	1/S	1/V
1	15	0.485601	0.066667	2.059305	0.791185	0.066667	1.263928
2	7.5	0.466321	0.133333	2.144444	0.705234	0.133333	1.417969
3	5	0.359079	0.2	2.784899	0.49146	0.2	2.034753
4	1.5	0.350645	0.666667	2.85189	0.440771	0.666667	2.26875
5	0.15	0.306061	6.66667	3.267323	0.254545	6.66667	3.928571

The Km values of PO for DL-Dopa was found to be 3.03 and 2.0 mM in normal and virosed population, respectively, showed 51.5 per cent decrease in Km (Table 12). Similarly, Vmax values were 0.571 and 0.832 ΔA/mg/min in normal and virosed population respectively, showed 45.70 per cent increase as compared to normal strain. Thus, it was found that there was increase in velocity of enzyme (increase in Vmax) and increase in affinity of substrate towards PO (decrease in Km).

Table 12: Michaelis constant (Km) and maximum velocity (Vmax) of phenoloxidase from normal and virosed V instar larvae of *H. armigera*.

Sr. No.	Substrate	Normal larvae		Virosed larvae		Percent decrease	Percent increase
		Km (mM)	Vmax (ΔA/mg/min)	Km (mM)	Vmax (ΔA/mg/min)	Km (%)	Vmax (%)
1	L-Tyrosine	1.36	0.333	1.21	0.377	12.39	13.21
2	L-Dopa	2.85	0.667	1.25	0.832	128	24.73
3	4-Methylcatecholin	5.405	0.454	1.66	0.667	225.60	46.91
4	Dopamine	6.25	1.053	5.55	1.587	12.61	50.71
5	DL-Dopa	3.03	0.571	2.00	0.832	51.5	45.70

4.3.2 Substrate Specificity

Substrate specificity was determined by studying haemolymph phenoloxidase conspiring from normal and virosed larvae of *H. armigera* with five different substrates viz., L-Tyrosine L-3-4 Dihydroxy phenylalanine (L- DOPA), 4-Mthylbrenzcatecholin, Dopamine Hydrochloride 3-hydroxytyramine chloride (Dopamine) and DL-3-4- Dihydroxy phenylalanine (DL-DOPA), at the highest concentration of substrate i.e. 15 mM used. During the course of study, same protocol of microplate assay was followed.

Enzyme activity was determined by performing the microplate assay with 50 μl of haemolymph obtained from normal and virosed larvae of *H. armigera*. The results are presented in Table 13 and depicted in Fig. 14. The data revealed that maximum phenoloxidase activity was observed in the substrate Dopamine at specific concentration of 15 mM. The substrate Dopamine exhibited PO activity of 656.165 Unit/mg protein/min in normal larvae and of 902.659 Unit/mg protein/min in virosed larvae. It was followed by L-Dopa, which showed PO activity of 562.141 and 874.115 Unit/mg protein/min by normal and virosed larvae respectively. DL-Dopa showed 482.104 Unit/mg protein/min PO activity towards haemolymph of normal larvae, whereas, in case of virosed larvae PO activity was 685.521 Unit/mg protein/min. Somewhat lower activity was recorded by L-Tyrosine and Metylcatecholin at exhibited PO activity of 308.63 and 292.944 Unit/mg protein/min to the normal larvae and 193.285 and 471.043 Unit/mg protein/min activity by virosed larvae, respectively.

Table 13: Substrate specificity of phenoloxidase (PO) from normal and virosed V instar larvae of *H, armigera*

Sr. No.	Substrate	Enzyme activity Unit/mg protein/min		Percent increase in PO Activity
		Normal	**Virosed**	
1	L-Tyrosine	308.63 ± 2.00	193.28 ±3.23	N.D*
2	Dopamine	656.16 ± 3.29	902.65 ±2.60	55.30
3	L-Dopa	562.14 ± 2 .09	874.11 ±2.44	50.10
4	4-Methylcatecholin	292.94 ± 2.40	471.04 ±2.34	20.10
5	DL-Dopa	482.10 ± 2.09	685.52 ±3.02	42.10

* N.D- Not Detected

The data on increase in PO activity in virosed larvae as compared to normal larvae are presented in Table 13 and depicted in Fig.14. Maximum per cent increase in PO activity was recorded in Dopamine i.e. 55.3 per cent followed by L-Dopa and DL-Dopa with 50.1 and 42.1 per cent increase in PO activity, whereas, least increase in PO activity was recorded in 4-Methylcatecholin (20.1 per cent) and L-Tyrosine exhibited decrease in PO activity.

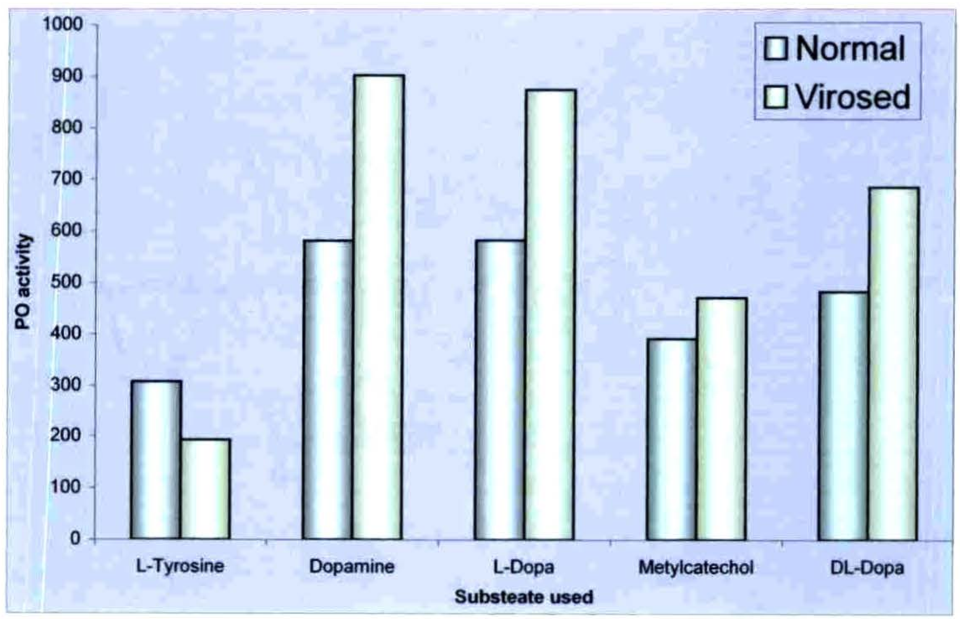


Fig. 14: Substrate specificity of phenoloxidase (PO) from normal and virosed V instar larvae of *H. armigera*

4.3.3 Optimum Temperature

To ascertain the effect of temperature on normal and virosed larvae of *H.armigera*, the following studies were conducted to determine the change in the PO activity in normal and virosed larvae due to change in temperature. The data presented in Table 14 and depicted in Fig. 15 showed that the PO activity of 822.34, 822.34, 1356.20, 1448.11, 641.73, 528.74, 180.70 and 5.90 Unit/ mg protein/min at 10, 20, 30, 40, 50, 60, 70 and 80 °C, respectively was obtained in normal larvae. It was observed that enzyme activity increases with the increase in the temperature up to 40^{0}C and maximum PO activity was observed at 40 °C (1448.11). Similar trend of PO activity was also observed in virosed larvae, which showed 1640.491 Unit/ mg protein/min PO activity at 40 °C. The PO activity both from normal and virosed haemolymph decreased after 50^{0}C and reached to minimum of 5.90 Unit/ mg protein/min at 80^{0}C in normal and not detected in virosed larvae.

Table 14: Effect of temperature on haemolymph phenoloxidase activity from normal and virosed V instar larvae of *H.armigera*

Temp in ^{0}C	PO Activity (Unit/ mg protein/min) (Mean ± SE)	
	Normal	Virosed
10	0822.34 ±5.24	0816.01 ±3.50
20	1353.66 ±4.13	1016.98 ±5.48
30	1356.20 ±4.75	1633.75 ±3.17
40	1448.11 ±2.50	1640.49 ±5.00
50	0641.73 ±5.30	781.89 ±2.65
60	0528.74 ±4.37	712.99 ±4.18
70	0180.70 ±2.96	41.78 ±4.11
80	005.90 ±0.28	ND*

*ND- Not Detected

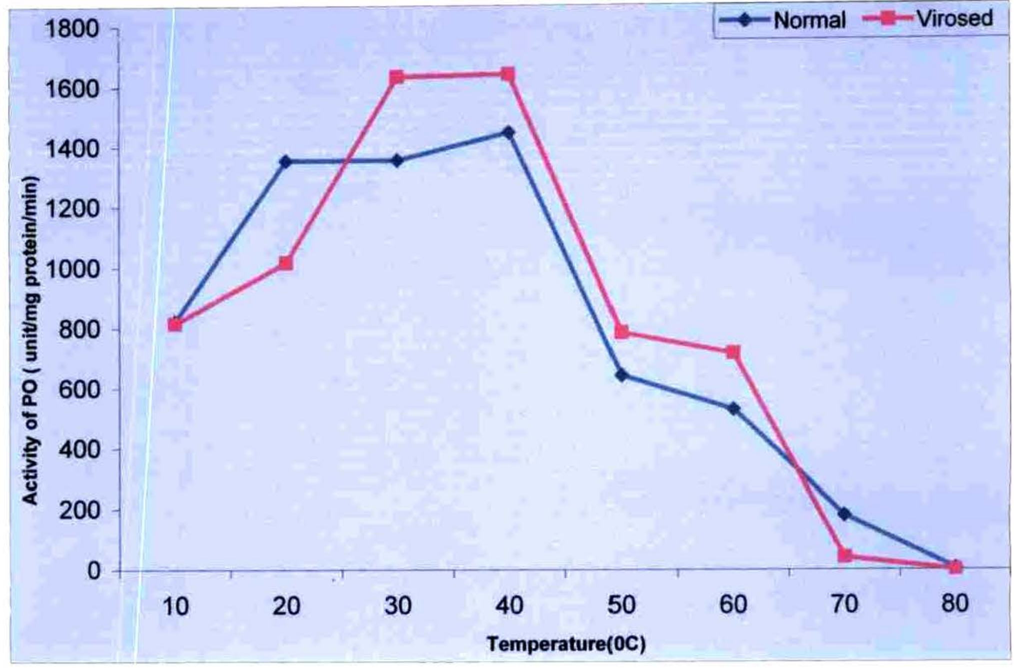


Fig. 15: Effect of temperature on haemolymph phenoloxidase activity from normal and virosed V instar larvae of *H.armigera*

4.3.4 Temperature stability

To assess the stability of PO activity on it's exposure to varied temperature and time period, the microplate assay by subjecting the incubation of haemolymph from normal and virosed larvae at specific temperature and time in thermal cycler were done. The details of the results are given in Table 15 and 16 and depicted in Fig.16 and 17. The PO from normal larvae of *H. armigera* showed almost 100 % enzyme activity at 10 ^{0}C, 20 ^{0}C and 30 ^{0}C up to 80 min. The PO activity was reduced to 83.21% after 50 min incubation at 40 ^{0}C. Further activity was found to decrease up to 76.93 % after 80 min of incubation at 40 ^{0}C. At 50 ^{0}C enzyme activity started to decrease after 30 min incubation, it decreased up to 62.59 % after 80 min incubation.

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 ^{0}C up to 80 min incubation. The enzyme activity was reduced up to 83.49 % at 40 ^{0}C after 50 min incubation. The reduction in PO activity at 40^{0}C was more than in normal larvae as it decreased up to 52.13 % after 80

min incubation. In virosed larvae, enzyme activity was decreased up to 45.93 % after 80 min incubation at 50 °C. Details are given in Table 15 & 16 and depicted in Fig. 16 &17.

Table 15: Thermal stability of the phenoloxidase from normal V instar larvae of *H.armigera*

Incubation Temperature / Time Interval (min)	10 °C	20 °C	30 °C	40 °C	50 °C
	Percent phenoloxidase activity				
10	99.56	99.56	99.56	100	100
20	99.56	100	100	100	100
30	100	100	100	100	100
40	100	100	100	100	77.35
50	100	100	100	83.21	68.07
60	100	100	100	80.25	65.28
70	100	100	100	80.25	63.26
80	100	100	100	76.93	62.59

Table 16: Thermal stability of the phenoloxidase from virosed V instar larvae of *H.armigera*

Incubation Temperature / Time Interval (min)	10°C	20 °C	30 °C	40 °C	50 °C
	Percent phenoloxidase activity				
10	99.56	99.56	99.56	100	100
20	99.56	100	100	100	100
30	100	100	100	100	100
40	100	100	100	100	73.50
50	100	100	100	83.49	70.50
60	100	100	100	78.76	68.36
70	100	100	100	54.50	50.73
80	100	100	100	52.13	45.93

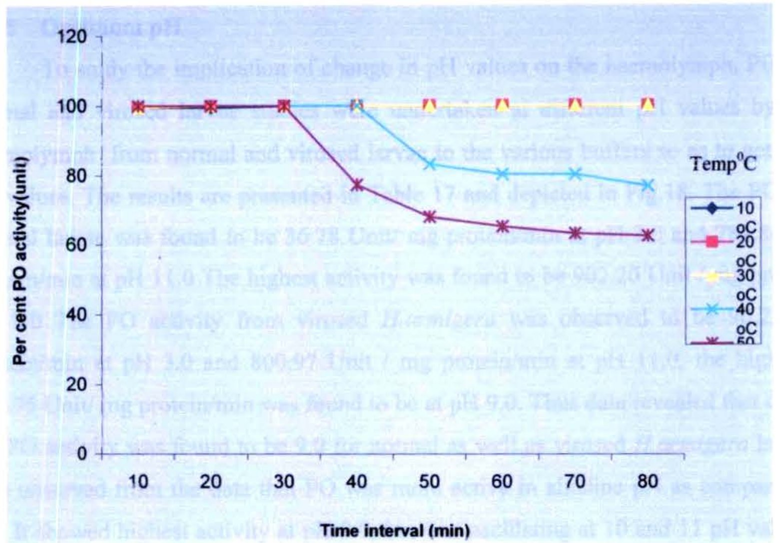


Fig. 16: Thermal stability of the phenoloxidase enzyme from normal V instar larvae of *H.armigera*

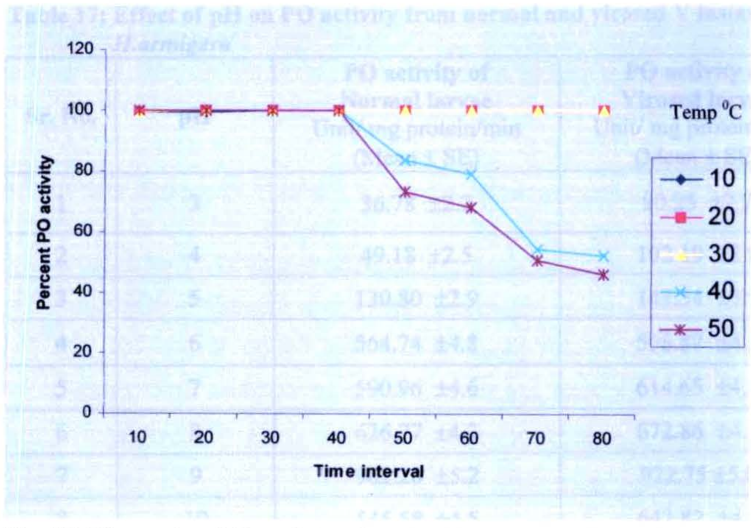


Fig. 17: Thermal stability of the phenoloxidase enzyme from virosed V instar larvae of *H.armigera*

4.3.5 Optimum pH

To study the implication of change in pH values on the haemolymph, PO activity of normal and virosed larvae studies were undertaken at different pH values by subjecting haemolymph from normal and virosed larvae to the various buffers so as to get the desired pH values. The results are presented in Table 17 and depicted in Fig.18. The PO activity in normal larvae was found to be 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 to be 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 to be at pH 9.0. Thus data revealed that optimum pH for PO activity was found to be 9.0 for normal as well as virosed *H.armigera* larvae. It was also observed from the data that PO was more active in alkaline pH as compared to acidic pH. It showed highest activity at pH 9.0, but was oscillating at 10 and 11 pH values, getting lowered at pH 10 and become appreciably active in alkaline buffers with pH of 11.0.

Table 17: Effect of pH on PO activity from normal and virosed V instar larvae *H.armigera*

Sr. No.	pH	**PO activity of Normal larvae** Unit/ mg protein/min (Mean ± SE)	**PO activity of Virosed larvae** Unit/ mg protein/min (Mean ± SE)
1	3	36.78 ±2.2	90.25 ±2.7
2	4	49.18 ±2.5	102.19 ±2.8
3	5	130.80 ±2.9	143.54 ±3.9
4	6	564.74 ±4.8	558.87 ±4.2
5	7	590.96 ±4.6	614.65 ±4.3
6	8	626.77 ±4.3	672.86 ±4.5
7	9	902.20 ±5.2	922.75 ±5.0
8	10	545.58 ±4.5	642.82 ±4.6
9	11	784.84 ±4.7	800.97 ±4.6

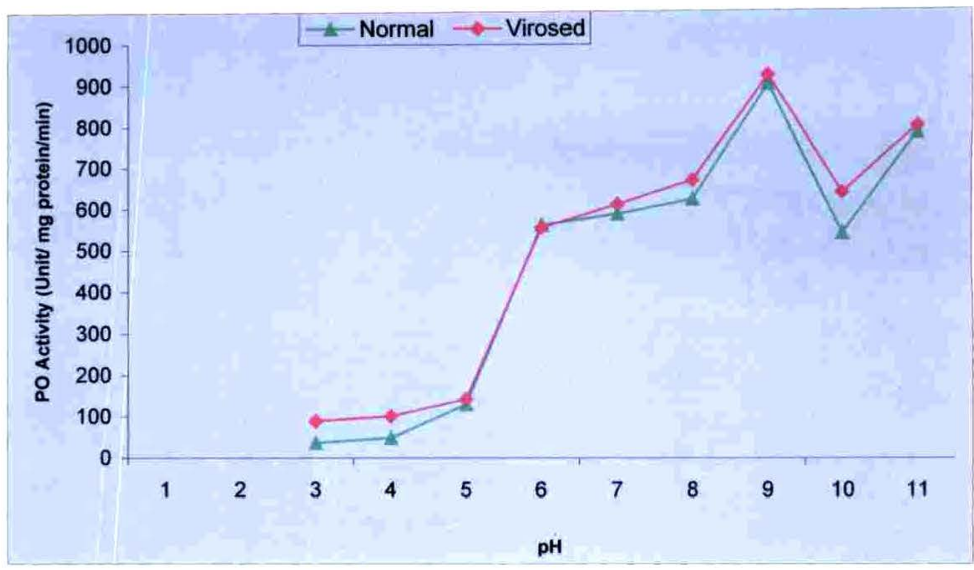


Fig. 18: Effect of pH on PO activity from normal and virosed V instar *H.armigera* larvae

4.4 Phenoloxidase activity and HaNPV toxicity in different chromotonal *H. armigera* larvae

The *H.armigera* larvae feeding on different host plants viz., cotton, chickpea, pigeon-pea and sunflower exhibited different chromotonal phenotypic relationship. So as to study the effect of these colourations on PO activity and toxicity due to HaNPV on different coloured larvae (green, brown and dark brown) of *H. armigera*, third and fourth instar were collected from the different host plants and were exposed to different dosages of HaNPV from 1 x10^3, 2 x10^3 to 1 x10^6, 1 x 10^6 POBs/ml and LC_{50} values were worked out. Similarly, phenoloxidase activities from these larvae were worked out and the data are presented in Table 18.

The lowest LC_{50} value of 1.1 x 10^4 POBs /larva was observed in 3rd instar green larva of *H. armigera*. However, there occurred tremendous increase in LC_{50} values in 4th instar larvae i.e. 4.2 x 10^5 POBs /larva. For light brown colour 3rd and 4th instar larvae LC_{50} values were 2.9 x 10^4 and 3.35 x 10^5 POBs /larva, respectively, indicating more or less similar susceptibility in both instar. This was also similar in case of dark brown larvae where in LC_{50} value were 2.7 x 10^4 and 4.17 x 10^5 POBs /larva.

Likewise, the PO activity from different colour larvae of *H.armigera* were also worked out. In green colour 3rd and 4th instar larvae, PO activity were 553.32 and 911.0 Unit/

mg protein /min, whereas, in light brown colour larvae,PO activity was found to be 516.82 and 821.03 Unit/ mg protein/min in 3rd and 4th instar larvae, respectively. In dark brown larvae of H. *armigera,* PO activity was found to be 446.44 and 824.50 Unit / mg protein /min

Table 18: Phenoloxidase and HaNPV toxicity from different chromotonal virosed V instar *H. armigera* larvae

Colour of larvae	POactivity Unit/ mg protein/min (Mean ±SE)		LC_{50} values POBs /larva	
	3rd instar	4th instar	3rd instar	4th instar
Green	553.32 ± 6.64	911.08 ± 7.40	1.1×10^4	4.2×10^5
Light brown	516.82 ± 5.82	821.03 ± 3.50	2.9×10^4	3.35×10^5
Dark brown	446.44 ± 6.68	824.50 ± 4.12	2.7×10^4	4.17×10^5

for 3rd and 4th instar, respectively. As such from this data, it was revealed that there was no correlation in colour of larvae HaNPV toxicity and phenoloxidase activity in this case

4.5 Inhibitor Specificity

It has been reported earlier that the PO activity can be inhibited by various inhibitors and hence, the study on PO inhibition was undertaken by using different inhibitors with various concentrations namely, EDTA (25 mM, 5 mM, 2.5 mM), PTU (15 mM, 7.5 mM, 1.5 mM), PMSF (25 mM, 12.5 mM, 1 mM), SDS (1%, 0.5%, 0.05%), DMSO (10%, 1%, 0.1%), STI (10mg, 1mg, 0.1mg), EGTA (25 mM, 12.5 mM, 5 mM) and Garlic extract (10%, 5%, 1%).

Microplate assay for PO were performed after 30 min of incubation by incubating 25 μl of different concentrations (as given with each inhibitor individually) of all above said inhibitors with 25μl of haemolymph from virosed larvae of *H. armigera* in each well, the data are presented in Table 19.

When compared with control having 100 per cent activity, the different inhibitors were given effective inhibition of PO at higher concentration except garlic extract. SDS was found to be the best inhibitor than all other inhibitors studied, it was followed by EGTA >PTU> EDTA > PMSF> DMSO > Garlic extract. The maximum inhibition was observed due to SDS at 1 % concentration (56.16 %) and was followed by 25mM concentration of

EGTA (24.67%), PTU (19.94%), EDTA (12.86%), PMSF (7.48%) and DMSO(7.34%). Whereas, STI didn't show any inhibition, at any of the concentration, while SDS was unable to inhibit PO at 0.10% and 0.50% concentration.

EGTA at a concentration of 2.5, 10 mM showed 5.90 and 5.38 % inhibition, PTU showed 9.8 and 11.2 % inhibition at 2.5 and 10 mM concentration, respectively. EDTA was found to inhibit 10.49 and 7.87 % of PO at the concentration of 10 mM and 2.5 mM, respectively. PMSF showed same phenoloxidase inhibition pattern, with 4.72 and 5.77 % inhibition at 10mM and 2.5 mM concentration. Like wise DMSO 10 % could inhibit PO 7.34 % and 5.24 % at 1 % concentration. Surprisingly Garlic extract was found to be inhibitor at lower concentration i.e. at 1 % (2.88 %) than at higher concentration of 5% and 10% whereas only 0.131 % and no inhibition was found.

Table 19: Effect of different inhibitors on phenoloxidase activity

Sr No	Concentration of Inhibitor	% Inhibition of PO	Sr No	Concentration of Inhibitor	% Inhibition of PO
1	EDTA (25mM)	12.86	5	EGTA (25mM)	24.67
	EDTA (10mM)	10.49		EGTA (10mM)	5.38
	EDTA (2.5mM)	7.87		EGTA (2.5mM)	5.90
2	STI (10mg)	NI*	6	PMSF (25mM)	7.48
	STI (1mg)	NI		PMSF (10mM)	4.72
	STI (0.1mg)	NI		PMSF (2.5mM)	5.77
3	PTU (25mM)	19.94	7	DMSO (10%)	7.34
	PTU (10mM)	11.02		DMSO (1%)	5.24
	PTU (0.5mM)	9.58		DMSO (0.10%)	NI
4	SDS (1%)	56.16	8	Garlic Extract (10%)	NI
	SDS (0.50%)	NI		Garlic Extract (5%)	0.131
	SDS (0.10%)	NI		Garlic Extract (1%)	2.88

***NI – Not Inhibited**

4.6 Electrophoretic study

Phenoloxidase isozyme pattern has been studied on native PAGE by using 10% running gel and 5% stacking gel. Gel was stained by using 40mg of L-Dopa in 100 ml of 0.1 M phosphate buffer (pH 6.5) that was prepared and filtered just before use. Crude haemolymph of normal and virosed larvae of *H. armigera* was loaded with 40 mg protein concentration.

Electrophoretograph (Plate 3) obtained after incubation of gel with L-Dopa revealed that two PO isozymes were present in haemolymph of normal and virosed *H armigera* larvae. In normal *H armigera* larvae, both isozymes (PON1 and PON2) migrate in close proximity with Rf values of 0.087 and 0.101, respectively (Plate 3).

Interestingly, haemolymph of virosed *H. armigera* also posses 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.

4.7 Effect of different phenoloxidase inhibitors in combination with HaNPV on *H.armigera* larvae

From the inhibitor study reported earlier, the inhibitor which were found effective in inhibiting the phenoloxidase activity *in vitro* were taken in different concentrations and incorporated in the semi-synthetic diet and mortality were worked out and compared. The inhibitor SDS (1%), EGTA (25mM) and PTU (25mM) were found to be effective in inhibiting phenoloxidase *in vitro*. But to know how they work *in vivo*, 4th instar *H. armigera* larvae were exposed to different concentration of HaNPV along with inhibitors and LC_{50} values were worked out and compared with control.

The data revealed that the LC_{50} values for inhibitor plus HaNPV incorporated artificial diet with SDS (1%) was 3.4×10^5 POBs/larvae, and for PTU (25mM) was 3.1×10^5 POBs /larvae, whereas in control i.e. without any inhibitor the LC_{50} value was found to be 3.7×10^5 POBs /larvae. The LC_{50} values of 3.9×10^5 POBs /larvae indicated very little or no effect of EGTA (25mM) when incorporated into the HaNPV. Simultaneously, the effect of these inhibitors individually on mortality of *H.armigera* was also tested and no effect of these inhibitors was found at the concentration tested.

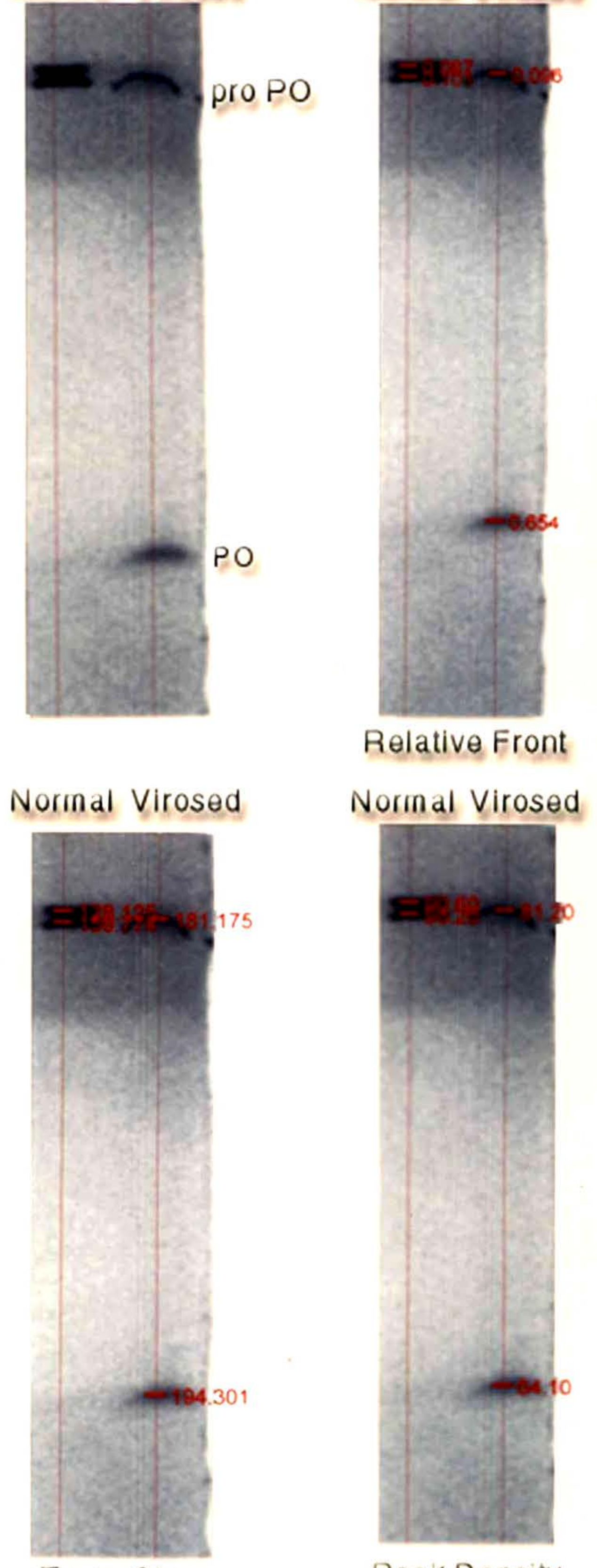


Plate 3. Haemolymph PO isozyme pattern from Normal and Virosed larvae of *H. armigera*

4.8 Total Haemocyte count

The effect of HaNPV on the total haemocyte count was assayed through the total haemocyte count (THC) from normal and virosed larvae of *H. armigera* by collecting 20µl of freshly drawn haemolymph in to 180 µl of anticoagulant. From this 200µl, one drop was kept on the haemocytometer and count was taken. The data presented in Table 20 and depicted in Fig.19, indicated that in 3rd instar larvae of *H. armigera,* THC of both virosed and normal larvae were same at first day after infection. Later on these increased in normal larvae up to forth day after infection, whereas in case of virosed larvae, THC increased up to second day, but thereafter, it declined continuously.

Table 20: Total haemocyte count mm^3 of haemolymph in normal and virosed larvae of *H. armigera*

Days after Treatment	3rd instar *H. armigera*		5th instar *H. armigera*	
	Normal	Virosed	Normal	Virosed
1st Day	30700 ± 545.89	30500 ± 649.61	37780 ± 614.00	37900 ± 350.71
2nd Day	33100 ± 665.58	32900 ± 450.55	37700 ± 506.95	36200 ± 559.46
3rd Day	35080 ± 481.66	30700 ± 376.82	35700 ± 690.65	34200 ± 581.37
4th Day	36580 ± 420.71	29400 ± 466.90	33600 ± 559.46	30400 ± 678.23

As far as 5th instar larvae of *H.armigera* were concerned, THC was found almost the same in both normal and virosed larvae at first day after infection but thereafter, it declined consistently till the fourth day after infection. However, the rate of decline was more in virosed larvae as compared to normal

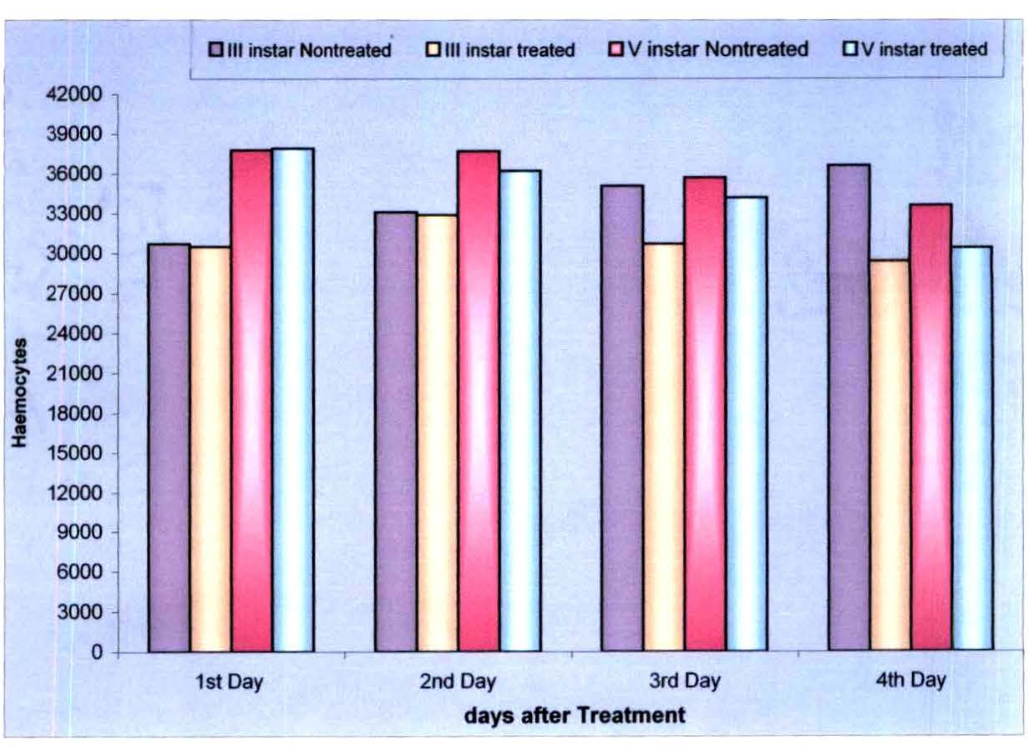
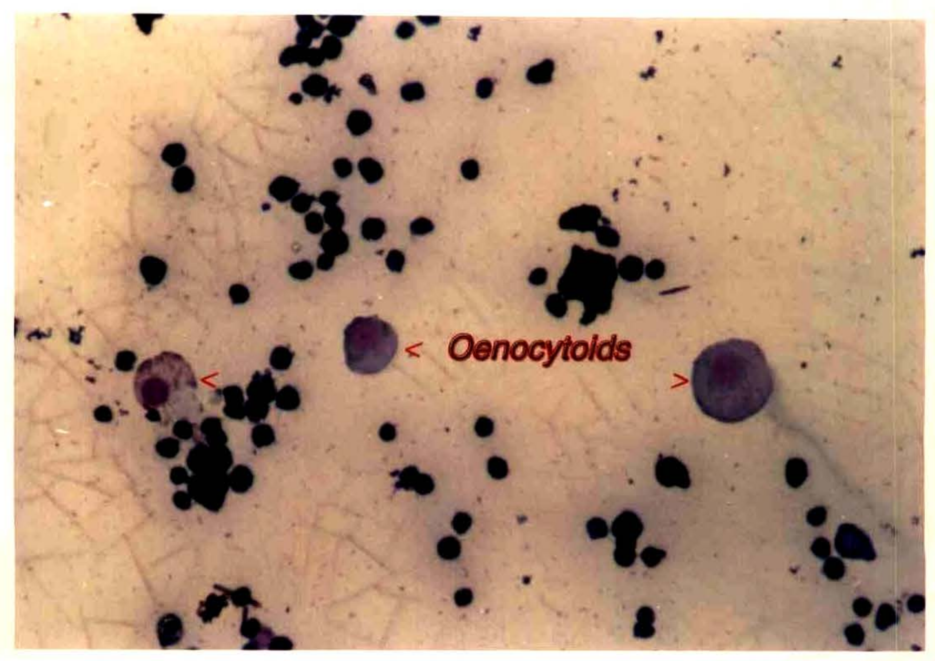


Fig.19: Total haemocyte count mm^3 of haemolymph in normal and virosed larvae of *H. armigera*



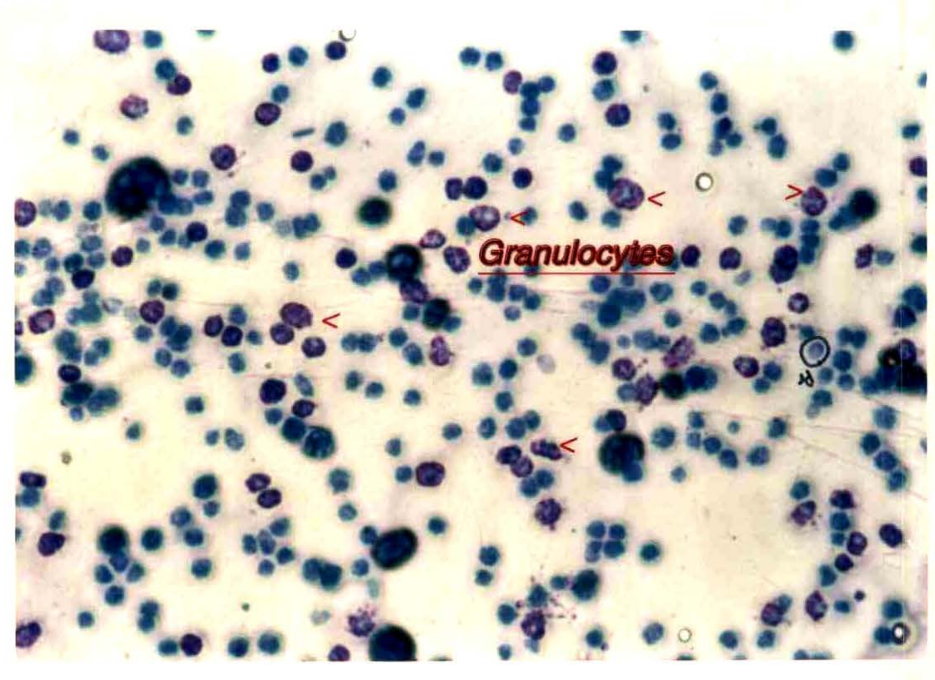
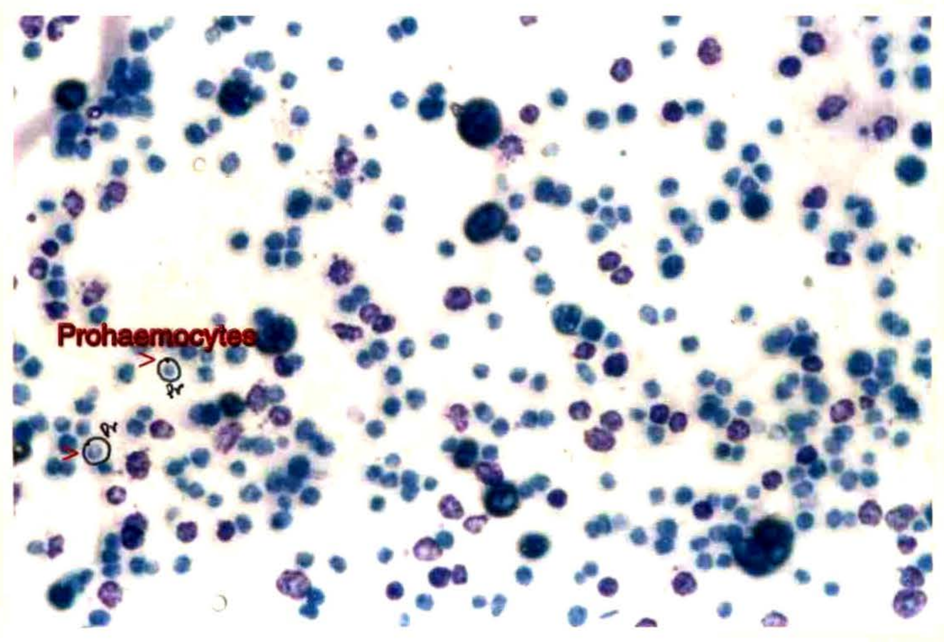


Plate 4. Haemocytes from *H. armigera*



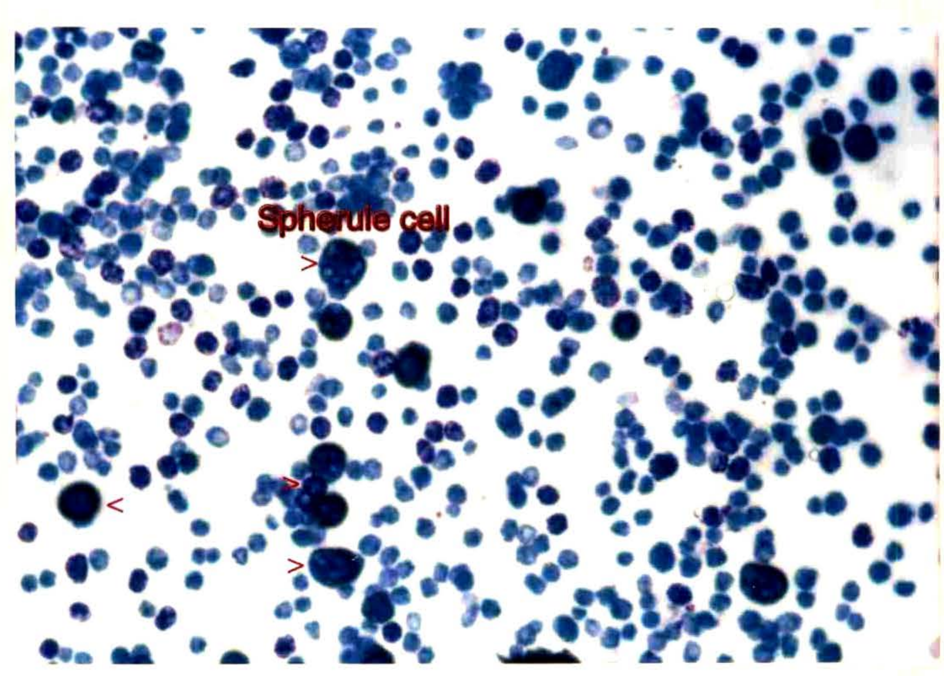


Plate 5. Haemocytes from *H. armigera*

CHAPTER V

DISCUSSION

The experimental findings pertaining to the studies on phenoloxidase and melanization in *Helicoverpa armigera* exposed to HaNPV, described earlier have been discussed herewith in light of the earlier research findings.

5.1 Age related bioassay of *H.armigera* with HaNPV

The results of the laboratory experiment on bioassay of HaNPV for determination of LC_{50} value for second, third, fourth and fifth instar larvae,presented under section 4.1 and Table 4, are discussed herewith. The LC_{50} of HaNPV for different instar *H.armigera* larvae were different in terms of POBs/ml. For second instar larvae, it was found to be 2.1×10^3 POBs/ml while it measured 1.0×10^4 POBs/ml for third instar larva.

The LC_{50} values were found to increase with fourth and fifth instar larvae, the value being 3.4×10^5 and 2×10^6 POBs/ml, respectively. The higher LC_{50} value for fourth and fifth instar clearly indicated the requirement of higher concentration of POB for their 50 per cent mortality. Thus, the data revealed that second instar larvae were 4.91 time more susceptible than 3rd instar larvae and 161.73 and 690.57 times more susceptible than fourth and fifth instar larvae, respectively. This means second instar larvae proved to be more susceptible and most vulnerable to virus and required lowest concentration of POBs.

Similarly, the third instar larvae were 32.97 and 195.83 time more susceptible than fourth and fifth instar larvae, respectively whereas, the fourth instar larvae showed 15.9 times more susceptibility over fifth instar. Slope for concentration and mortality relationship varying from 0.72-0.47 was in the range as mentioned by Burges and Thompson (1971). There was no significant difference in the slope values of the concentration mortality regression line indicating that the insect population was responding in similar homogenous manner.

The slope value of matured larvae was lower than those for immature larvae, the shift in probit line reflecting that the older larvae display less variation in response to HaNPV. The

drop in b value showed that the increase in mortality due to increased concentration, decreased with host age. Thus the susceptibility decreased as age of larvae increased.

Age developmental resistance has been reported in many species of Lepidopteron larvae challenged with baculovirues. Stairs (1965) observed that first instar larvae of forest tent caterpillar *Malacosoma disstria* (Hub) were 1000 times more susceptible than third instar larvae and about 68000 time susceptible than fourth instar larvae. Similarly, Komolpith and Ramarishanan (1975) conducted bioassay of *S. litura* and reported LC_{50} value for 4, 5, and 7-day-old larvae, which were 4.677,13.70,37.240 x 10^6 POBs/ml, respectively. Bucher and Turnock (1983) also reported LC_{50} dose of 235, 444, 519 and 14130 POBs/larvae for second, third, fourth and fifth instar larvae, respectively.

Similarly, Gitanjali Jayachandran and Chaudhari (1996) found that the LC_{50} value increased with host age which were 7.57 x 10^4 , 3.33 x10^5, 2.23 x 10^7 and 5.08 x 10^8 POBs/ml for 3,5,8 and 10 days old larvae, respectively. Likewise Kencharaddi and Jayaramaiah (1997) also reported that LD_{50} values were 2.9 x 10^4, 5.33 x 10^4 and 2.7 x 10^5 POBs/ml for first, third and fifth instar larvae of *H.armigera.*

Studies on the virulence of HaNPV were performed by Datkhile (2000) and found that the LD_{50} of NPV for 2nd, 3rd, 4th and 5th instar were 0.30 x 10^3, 4.42 x 10^3, 6.13 x 10^3 and 47.6 x 10^3 POBs/larvae, respectively. The susceptibility of 2nd instar *H. armigera* larvae was found to be 4.7,20.25 and 157.1 times more than third, fourth and fifth instar larvae. Also Monobrullah and Nagata (2000) observed the developmental resistance in larvae of *S. litura.* The LD_{50} value of second, third, fourth and fifth instar larvae were found to be 224, 806, 20692 and 519381 POBs/larvae, respectively. Thus, the second instar larvae were about 305, 92, 2314 fold more susceptible than third, fourth and fifth instar larvae, respectively.

In the same way Kalia and Chaudhari (2001) found that LC_{50} value increased with age in bioassay of *H. armigera* against HaNPV. The LC_{50} values were 16.93, 4.54 x 10^2, and 5.67 x 10^3 and 2.06 x 10^5 OB ml^{-1} for 1,3,8 and 10 day old larvae, respectively. One day old larvae were 26.76,334, and 12100 times more susceptible than 3, 5, and 8 day old larvae, respectively. Recently, Vinodkumary and Singh (2002), reported that the susceptibility of the larvae was negatively correlated with larval period of development. LC_{50} value for 1,3,5,8and 10 day old larvae were 8.2 x 10^3, 2.22 x 10^4, 2.273 x 10^6 5.08 x $10^8$and 1.39 x 10^{10} POBs/ml

and one day old larvae were 2.7, 277.0, 61951.2 times more susceptible than three, five and eight day old larvae, respectively.

Most of the published data indicant that the decrease in susceptibility was largely linked to increased body weight as reported by Whitlock, (1977) and Evans, (1981). In *M. brassicae* body weight increased 306 times whereas, the LC_{50} was hiked by 6710 time (Evans, 1981), so increase in body weight is not the only reason behind this phenomenon other's may be there. As, in case of matured *H. armigera* there was no change in susceptibility when the virus was administrated into haemolymph (Teake *et al.*, 1986). Their finding showed that in *H. armigera* mature larvae the secondary cycle of replication, which is essential for the mortality of the host larvae, did not take place in the haemolymph. The physiological changes associated with pupation may not allow infection at this late developmental stage or the virus did not have enough time for replication so as to cause mortality in insect. Pupation takes place before the virus could exert its influence on larvae indicating apparent total resistance of older larvae as reported by Gitanjali Jayachandran and Chaudhari (1996).

Whereas, Prasad and Ramakrishinan (1993) reported various pre-gut and post-gut factors as possible candidates for conferring resistance to virus infection in larvae older than eight days in *S. litura.*

Gut appeared to act as a barrier to infection in late stage of larval development of *S. litura.* Larvae were not susceptible to infection *per os,* but highly susceptible to infection if a virus was injected in to the haemocoelof matured larvae as suggested by Monobrullah and Nagata (2000). Beyond this the gut barrier in *S. litura* appeared to have an important role in supplementing phenoloxidase (PO) system by retarding secondary NPV infection (by the virions that manage to gain entry) in the haemolymph as reported by Gitanjali Jayachandran *et al.*, (2000) and also found that PO level was high in older larvae as compared to younger one.

Thus, it seems that many factors are responsible for conferring the observed age related resistance viz., increase in biomass of mature larvae, secondly less time for infection as larvae proceed for pupation, change in receptor site in mid gut as pre – gut barrier and PO system/cascade as post gut barriers.

5.2 Determination of phenoloxidase in different age group of *H. armigera*

The haemolymph consists of plasma and haemocytes, in fluid plasma haemocytes or blood cells are suspended (Chapman, 1998). The results on the determination of phenoloxidase in different age groups of *H. armigera* presented in Table 5 & 6 and depicted in Fig. 2 & 3, are discussed below. In case of the normal 3rd instar larvae, the PO activity in cellular fraction was found to increase from 322.99 Unit/mg protein/min. to 411.32 μM/protein/min during 72 hr after initiation of infection. Whereas, in case of plasma fraction PO activity was not detected initially and it remained so up to 48 hr but at 72 hr it was found to be152.63 Unit/mg protein/min.

Likewise in case of virosed larvae for the 3rd instar, PO activity recorded in the cellular fraction was 325.41 μM/protein/min initially at 0 hr, and reached to 902.24 Unit/mg protein/min after 72 hr. As against this the plasma fraction of the same group recorded no PO activity initially at 0 hr however, after 24 hr it was found to be 21.62 Unit/mg protein/min and increasing trend continued up to 72 hr (272.20 Unit/mg protein/min)

In second group i.e. in normal 5th instar larvae of *H. armigera*, the PO activity in cellular fraction was found to be 427.04 Unit/mg protein/min at 0 hr and it increased during 48 hr to 506.70 Unit/mg protein/min. But later on decreased to level of 431.49 Unit/mg protein/min at 72 hr.Whereas in plasma fraction of same group PO activity at 0 hr was found to be 229.18 Unit/mg protein/min and later found to increase up to 392.04 Unit/mg protein/min at 72 hr.

Cellular fraction of virosed 5th instar larvae of *H. armigera* had a higher PO enzyme level at 0 hr i.e. 645.71 Unit/mg protein/min which oscillated to the extent of 889.89 Unit/mg protein/min at 48 hr and later on decreased up to 508.99 Unit/mg protein/min at 72 hr. However, in case of plasma fraction of virosed 5th instar larvae, the enzyme activity at 0 hr was 234.32 Unit/mg protein/min level and found to increase considerably up to 968.34 Unit/mg protein/min as the age of larvae increased

So in general, the PO activity in plasma fraction of both virosed and normal larvae of *H. armigera* goes on increasing, however in case of cellular fraction it was found to increase in 3rd instar in virosed and normal larvae but in 5th instar the PO activity in cellular fraction was found to decrease as the age increased, whereas in plasma fraction the quantity of PO in

5th instar larvae was more as compared to 3rd instar larvae of *H. armigera*. The higher activities of PO in NPV treated larvae seem to attribute with on set of NPV infection.

Bidochka *et al* (1989) suggested that PO is not bound to haemocyte membrane and probably existed in the hemocytic cytoplasm or haemolymph plasma in Acridid grasshopper from the subfamily Melanoplinae and Oedipodinae. Further they suggest that difference in PO activity may reflect differences on immune response to various pathogens, the different methods of pathogen disease formation and the subsequent response of PO system.

Miranpuri *et al.*, (1992) found that in grasshopper *Melanoplus sanuinipes,* PO activity was at peak in infected grasshopper due to infection by entomopoxvirus 4 days post infection. Similarly, Nigam *et al.*, (1997) reported that Phenoloxidase activity of refractory tsetse flies *Glossina palpalis palpalis* were always higher than those obtained from susceptible *G. morsitans morsitans*. Whereas, Gillespie (2000) found the initial increase in the activity of phenoloxidase of desert locust *Schistocerca gregaria* during mycosis of the *Metarhizium anisopilae* var. *acridium* and later on decrease during the course of infection, for this they attributed the initial recognition of the fungus and fungus derived metabolites, which later on get altered leading to overcoming of immune system.

Likewise Hung and Boucias (1996) reported that phenoloxidase titer in *Spodoptera exigua* larvae infected with *Beauveria bassiana* at 24 hr post challenged, in plasma and haemocytes lysate (HL) was comparable with control. But the PO titer in HL sampled from infected larvae decreased seven fold whereas plasma titer increased at 48 – 60 hr post challenged which is in conformity of our finding.

Similarly, Gitanjali Jayachandran *et al.*, (2000) reported increase in phenoloxidase activity in 5 day old larvae of the *S. litura* haemocytes at 48 hr in response to NPV treatment and there was steady decline in PO activity of haemocytes in 10 day old larvae. As against this, the PO activity of the plasma fraction increased in 5 and 10 day old larvae. the PO activity of plasma increased with the age but magnitude of increase grows with NPV treatment. The present findingsare in conformity which indicated similar shift in the PO of *H. armigera* as was observed in *S.litura* in response to NPV from the cell fraction to the plasma fraction in 10 day old larvae.

Also Kalia *et al.,* (2001) found that PO activity of plasma increased with the age in *H.armigera.* PO activity of haemocytes increased up to two days in response to NPV infection in 5 day old larvae but declined in 10 day old larvae. Whereas increase in PO activity of plasma fraction was reported in both 5 as well as 10 day old larvae, with larger magnitude of PO activity in 10 day old larvae. All the above findings are comparable with the present studies.

5.3 Characterization of phenoloxidase from normal and virosed larvae of *H. armigera*

Enzymatic characterization of PO is important in that it provides better understanding of this enzyme, which is needed for melanization and sclerotization reaction. In addition, melanization may be important in the insect defense response.

5.3.1 Kinetics of phenoloxidase

In present study, crude haemolymph was used for characterization of phenoloxidase (PO). 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 total PO.

The kinetics of phenoloxidase was studied by using different substrates viz., L-Tyrosine, L-Dopa, Methylcatechol, Dopamine and DL-Dopa for measuring the phenoloxidase activity. Lineweaver-Burk double reciprocal plot was drawn by plotting concentration of substrate and variable absorbance due to the different substrates. Michaelis constant (Km) and maximum velocity (Vmax) was deduced for phenoloxidase from normal and virosed larvae of *H. armigera.*

Michaelis constant (Km) is a substrate concentration, which corresponds to half Vmax and is a measure of affinity. If Km increases it means that the affinity of enzyme decreases towards substrate and reverse is true if Km decreases. During present study the Km of PO from virosed larvae decreased in comparison to normal larvae in all the five substrates. This means that the affinity of enzyme towards substrate increases in virosed larvae. Further, it indicated that most of the PO is free or activated due the infection of HaNPV. This occurred due to the likeliness of all tested substrate towards increase in enzyme.

Secondly, Maximum velocity (Vmax) is measure of titer of enzyme which denotes the maximum velocity and is in proportion of the increase or decrease of enzyme quantity, if Vmax increases the quantity of enzyme increases and vice versa. During present study the Vmax in virosed larvae increased over that of normal larvae indicating that amount of PO is more in virosed larvae due to HaNPV infection as compared to normal larvae or virosed larva have more active PO as compared to normal larvae. These higher levels of PO in haemolymph could lead to more rapid and vigorous melanization, encapsulation reaction in response to exposure to a pathogen (Reeson *et al.*, 1998)

The data presented in Table 12 indicates that, normal larvae PO had a Km of 1.36mM whereas 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 was found in normal and virosed larvae respectively, whereas Vmax was found to be 0.667 and 0.832 ΔA/mg protein /min. respectively. Similar observations were made by Lockey and Ourth (1992), who found that Km for *H. virescens* larval haemolymph PO was 2.25mM and Vmax was 0.235 ΔA/mg protein /min, when L-Dopa was used as a Substrate. Morgan *et al.*, (1990) also reported 2.2mM Km for integument PO of *M. sexta*. While, the Km of the haemolymph tyrosinase from *M. sexta* was 4.5mM (Aso *et al.*, 1984).

Same way Ashida and Dokhe (1980) reported that in *B.mori* haemolymph, PO had Km of 1.7mM, 1.8 mM and 2.0mM besides 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. Adamo (2004) also found the Km of 2.5mM and Vmax of 5mM/min for haemolymph PO of *Gryllus texensi* at pH 7.4. Similar results were also noticed by Siva-Jothy *et al.*, (2001) 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 haemolymph 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. Identical result regarding Km of virosed larvae were reported as for normal larvae whereas 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 of immune as compared to normal one contrary to our study wherein Vmax was found to be increasing in virosed larvae as compared to normal. This might be due to more active enzyme from virosed larvae as against normal larvae. Barrett and Anderson (1981) reported Km 0.22mM when Methylcatechol was used as a substrate in Blow fly *Calliphera vinica* for cuticle PO enzyme B and when L-Dopa was used as substrate Km found was to be 1.67mM.

During present study when Dopamine was used as a substrate Km was observed as 6.23mM and 5.55mM for normal and virosed larvae whereas Vmax was found to be 1.053 and 1.587 ΔA/mg protein /min respectively. Like wise 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, however they used purified PO for the study. When DL-Dopa was used as a substrate Km was found to be 3.03 mM and 2.00 mM, whereas, Vmax was found to be 0.571 and 0.832 ΔA/mg protein /min for normal and virosed larvae respectively and also reported that the substrates have inhibitory effects on the enzyme at high concentrations.

5.3.2 Substrate specificity

Results presented in Table 13 and depicted in Fig.14 revealed that amongst all the substrate tested, 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 haemolymph, which was followed by L-Dopa, DL-Dopa, Methylcatechol and L-Tyrosine.

Similar results were reported by Aspan *et al.*, (1995) who assayed substrate specificity of the pure protein by using different mono and diphenols and reported that tyramine and tyrosine were readily hydroxilated by the PO enzyme in cryfish. Further they reported that, enzyme oxidize o-diphenols and reported dopamine were the a best substrate for PO of *Pacifastacus leniusculus* followed by *N*-acetyldopamine, L-dihydroxyphenylalanine, 4-methylcatechol and catechol. Like wise Hall *et al.*(1995) showed that for *M.sexta* PO, N-β-alanyldopamine, Methylcatechol and dopamine as best substrate and Dopa, an oftenly used substrate for routine assay of insect PO proved to be poor for *M.sexta* haemolymph PO. Same way Chase *et al.*, (2000) found that N-acetyldopamine, 4-methylcatechal, dopamine were better substrates than Dopa while making assay of PO in

Sarophaga bullata. Further they opined that unlike mammalian tyrosinase insect PO prefer Dopamine better than Dopa likewise, PO is typical O- diphenol oxidase possessing monophenol monooxygenase activity. Also, Asano and Ashida (2001) studied substrate specificity for cuticular PO for *B.mori* and found that better substrate in descending order were dopamine, *N*-acetyldopamine, L-dopa, metylhydroquinone and hydroquinone.

Thus, present study revealed that dopamine was a best substrate than any other among the studied and is recommended for insect PO study instead of, oftenly used Dopa.

5.3.3 Optimum temperature

The results presented in Table 14 and depicted in Fig.15 indicated that PO activity from *H.armigera* changes with respect to change in temperature. It was observed that enzyme was optically active at 40^0C (1448.11 Unit/ mg protein/min) in normal as well as virosed larvae (1640.49 Unit/ mg protein/min), although PO enzyme activity was observed at wide range of temperature. Same way Bidochka *et al.*, (1989) found that PO activity of grasshoppers from subfamilies Melanoplinae and Oedipodinae was optimally active at temperature 37^0C. Lockey and Ourth (1992) also reported that optimum temperature for *H.virescens* was 45^0C. Possible denaturation and loss of active 3-D structure of PO enzyme occurs at temperature above 40^0C as activity was decreasing after 40^0C in virosed and normal larvae. Similar observations were made by Tsukamato *et al.*, (1986).

5.3.4 Temperature stability

The results presented in Table 15 & 16 and depicted in Fig 16 & 17 on *H.armigera* haemolymph temporal PO stability at different temperature indicated that the PO from normal larvae showed almost 100 % enzyme activity at 10 °C, 20 °C and 30°C up to 80 min. The PO activity was reduced to 83.21% after 50min incubation at 40 °C, further activity was found to decrease up to 76.93 % after 80 min of incubation at 40 °C. At 50°C, activity decreased up to 62.59 % after 80 min incubation.

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 °C up to 80 min intervals. The enzyme activity was reduced up to 83.49 % at 40 °C after 50 min incubation. At 40°C enzyme activity was decreased up to 52.13 % and 45.93 % after 80 min incubation at 50 °C.

Like wise Ashida (1971) found that Pre-phenoloxidase from the haemolymph 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 housefly *Musca domastica* 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) found that in *Sarcophaga bullata*, the activated 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. In contrast heating the proenzyme for 10 min at 60^0C resulted in 50 per cent loss of its activity and heating at 70^0C for same time resulted in 100 per cent loss of its activity. The present findings are in conformity of the same (Table 14) where drastic reduction in PO activity was observed at 70^0C both for normal and virosed larvae of *H. armigera.*

So phenoloxidase from normal and virosed larvae of *H.armigera*, was stable up to 40^0C for about 40 minutes and 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.

5.3.5 Optimum pH

The results on the optimum pH experiment showed that in normal *H.armigera*, the PO activity was 48.62 Unit/ mg protein/min at pH 3.0 and 802.09 Unit / mg protein/min at pH 11.0. The highest activity was found to be 926.33 Unit / mg protein/min at pH 9.0. The PO activity from virosed *H.armigera* was observed to be 52.43 Unit/ mg protein/min at pH 3.0 and 764.56 Unit / mg protein/min at pH 11.0, the highest activity 879.67 Unit/ mg protein/min was found to be at pH 9.0.

Thus the result revealed that optimum pH for PO activity was found to be 9.0 for normal and virosed *H.armigera* larvae [Table 17, Fig.18]. It was also observed from the data that PO was more active in alkaline pH as compared to acidic pH. Like wise Ashida (1971) found that Pre-phenoloxidase from the haemolymph 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) reported that phenoloxidase from the housefly *Musca*

domastica was stable at pH 9.0, whereas purified latent PO was stable at pH 6.0. At pH 5.0 the remaining activity was about 25 % whereas at pH 8.0 the remaining activity was more than 30 % but was accompanied by slight activation.

While Bidochka *et al.,* (1989) found that PO activity of grasshoppers from subfamilies Melanoplinae and Oedipodinae had a pH optimum of 7.3. Whereas, Lockey and Ourth (1992) found that optimum pH for *H. virescens* larval haemolymph PO was 9.0.While Hung and Boucias (1996) found that optimum pH for naïve and *Beauveria bassiana* infected *S. exigua* larvae in haemolymph PO was 7.0. Whereas at pH 6.0 and above pH 8.0, the PO activity was suppressed and at pH 4.0, no PO activity was detected. Same way Chase *et al.,* (2000) also found that in *Sarcophaga bullata*the optimum pH for PO was 7.0.

So it is suggested that phenoloxidase from normal and virosed larvae of *H.armigera* like most other insect was active at alkaline pH around pH 9.0 than acidic even at pH 7.0 and below.

5.4 Phenoloxidase activity and HaNPV toxicity in different chromotonal *H. armigera* larvae

The results on determination of HaNPV toxicity and phenoloxidase from different colour larvae of *H. armigera* presented in Table 18 indicated that the LC_{50} value for 3rd instar green larva of *H. armigera* was 1.1 x 10^4 POB/larva i.e. minimum than for any other 3rd instar, indicating it's more susceptibility, however, it was not so for the 4th instar and for 4th instar larvae value was 4.2 x 10^5 POB/larva. For light brown colour larvae, LC_{50} values were 2.9 x 10^4 and 3.35 x 10^5 POB/larva respectively, similarly in case of dark brown 3rd instar larvae, LC_{50} value was 2.7 x 10^4 and for 4th instar larvae, value was 4.17 x 10^5 POB/larva. Thus, there was no consistency in LC_{50} due to dark pigmentation. This was more clear from PO activity.

PO activity in green colour larvae in 3rd and 4th instar were 553.32 and 911.0 Unit/ mg protein /min whereas, in light brown colour larvae PO activity was found 516.82 Unit/ mg protein/min in 3rd instar whereas it was 821.03 Unit/ mg protein/min in 4th instar. Likewise, in dark brown larvae in 3rd instar *H. armigera* PO activity was found to be 446.44 Unit/ mg protein/min and in 4th instar, the activity was 824.50 Unit/ mg protein/min. As such from this data, it was revealed that there was no relation between the colour of larvae, HaNPV toxicity and phenoloxidase activity.

While Reeson *et al.*, (1998) reported that the larvae of *S.litura* reared in crowded condition were black by the third instars and those reared in isolation develop green/brown coloration. These changes were attributed to have evolved in response to increased intra specific competition for food or density dependences predation pressure or in responses to the pathogen at high population densities, which was not true in present case. Likewise they observed overall mortality to the doses of NPV was 70% for green solitaries compared with 59% for black solitaries and 42% for crowded reared larvae. PO activity was significantly higher in solitary black and crowded (black) than in green larvae. Present findings differ from them in that, no correlation in colour of larvae and HaNPV toxicity was observed, which might be due to the fact that we reared *H.armigera* individually, whereas they reared *S.litura* in group, because, high density phase will show greater resistance to pathogen than low density phase

Present study, revealed no correlation in colour of larvae and phenoloxidase activity as was exhibited in *S. litura* (Reeson *et al* 1998) where larvae reared at a high density were found to be considerably more resistant to nuclear polyhedrosis virus than those reared in isolation. The conspicuous feature of high density phase was cuticular melanization. As melanization is controlled by the PO enzyme system, which is also involved in the immune responses, suggesting possible mechanism for increased resistance at high population density. However, the difference in melanization in *H. armigera* can not attributed to the density not to the host on which these are grown as against the increased resistances in melanize form at high population density than in non- melanize forms independent of rearing density which did not hold good during present investigation as no such relationship existed between the colour of the larvae and PO activity. Whereas, Reeson *et al.*, (1998) found that haemolymph phenoloxidase activity was correlated with cuticular melanization, thus present finding did not provide evidence for a link between cuticular melanization and immunity in *H. armigera,* it might be due to the fact that we measured PO from haemolymph, however, it was found that the granular phenoloxidase responsible for cuticular melanization in *M. sexta* was distinct from cuticular wound-healing, PO and haemolymph PO as reported by Hiruma and Riddiford, (1988). Reeson *et al.*, (1998) also suggested higher PO level as responsible for the cuticular melanization i.e. characteristic of high density form of many polyphonic species. However, this was not true in present finding as PO activity was comparatively less

in brown larvae than green one and hence the cuticular melanization as correlated with PO in haemolymph was not supported by present finding. However, they also did not observed significantly higher PO activity between the solitary black and crowded (black) larvae. This observation corroborates the present finding wherein not much difference was observed in PO activity of brown and dark brown larvae virosed with HaNPV. They opined that the cuticular PO might have imported in resisting pathogen that penetrate through cuticle such as entomopathogenic fungus.

However the route of infection of the HaNPV used in this study is through the midgut, so higher PO level in the cuticle should not in them affect susceptibility. Therefore the PO levels in midgut cell and haemolymph will have greater bearing on the infection taking hold as suggested earlier that for most of the Lepidopteran NPV's, initial viral replication occurs in the cell lining of the midgut and the virus then spread to other tissue via the haemolymph (Federici, 1997). So PO level in midgut lining and haemolymph had a greater impact on the infection of NPV. As the 2nd instar onwards *H. armigera* was mostly solitary, therefore, the cuticular melanization may not be attributed density as with many polyphonic species.

5.5 Inhibitor specificity

The result on PO inhibitor specificity are presented in Table 19 which indicated that 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) reported similar type of result in Blow fly *Calliphera vinica.* Cuticle PO enzyme 'A' was inhibited by Thiouria, Phenylthiouria but less so by sodium azide and NaF. Same way Anderson *et al.*, (1989) reported that PPO activation showed a bell-shaped dependence on EDTA or EGTA concentration with activation below and inhibition above 2mM.

While Bidochka *et al.*, (1989) found that the compound KCN and NaCN had inhibited PO activity of grasshoppers from subfamilies Melanoplinae and Oedipodinae but EDTA showed no effect.

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

Also Asano and Ashida (2001) found that cuticular PO in *B.mori* was completely inhibited by 1mM of phenylthiourea. Whereas, Mantawy and Mahmoud (2002) 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.

This shows that in *H. armigera,* detergent such as sodium dodecyl sulphate (SDS) and chelating agent like EDTA, EGTA or serine protease inhibitors Phenylthiourea were found to inhibit PO activity; these are believed to block the activation of Pro-PO to PO.

5.6 Electrophoretic study

Electrophoretogram obtained after incubation of gel with L-Dopa reveled two PO isozymes in haemolymph of normal and virosed *H armigera* larvae. In normal *H armigera* larvae both isozymes (PON1 and PON2) migrate in close proximity and possess higher molecular weight.

Whereas haemolymph of virosed *H. armigera* possess two PO isozymes (POV1 and POV2). Amongst it POV1 possess Rf value approximately similar to PON1 and PON2, which suggest that it possess more or less same molecular weight. All high molecular weight PO isozymes (PON1, PON2 and POV1) can be of inactive ProPO enzyme. Unlike POV1, POV2 possess very small Rf value and its smaller molecular weight is suggestive of activated phenoloxidase obtained after the cleavage of prophenoloxidse.

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

5.7 Effect of different phenoloxidase inhibitors in combination with HaNPV on *H. armigera* larvae

The results on effect of different phenoloxidase inhibitors in combination with HaNPV on *H.armigera* revealed that the LC_{50} values for inhibitor plus HaNPV incorporated artificial diet with SDS (1%) was 3.4 x 10^5 POB/larvae, for EGTA (25mM) 3.9 x 10^5 POB/larvae and for PTU was 3.1 x 10^5 POB/larvae, whereas in control i.e. without any inhibitor the LC_{50} value was found to be 3.7 x 10^5 POB/larvae. Effect was noted in mortality of larvae due to incorporation of these inhibitors with HaNPV, however, the intensity of effect was very little.

As observed in *in vitro*, inhibition of PO *in vivo* was not observed, indicating lack of effective inhibitors under *in vivo* condition. Inhibitors used in the present study were already characterized as protease inhibitor. However, there inability to work effectively in vivo condition might be due to the fact that the inhibitor application was made through the artificial diet which have entered through the digestive system of insect, it leaves question mark regarding the accsess of inhibitor to the PO haemolymph. As it may get digested in to insect midgut or may get eliminated as byproduct or might have been absorbed in some altered or ineffective form and thus might not have reached to it's specific target site i.e. haemolymph PO.

Recently Garcia *et al.*, (2004), first time demonstrated that eicosaenoid biosynthesis inhibitor phospholipase (PLA_2) inhibitor (dexamethasone), cyclooxygenase (COX) path way inhibitor (indomethacin) and non-selective lypoxygenase (LOX) inhibitor nordihydroguaiaretic acid (NDGA) attenuated the Pro-PO system in the haemolymph of *Rhodnius prolixus* infected with *Trypanosoma rangeli,* thus these results indicated that instead of using serine inhibitors to suppress PO, we have to use eicosaenoid inhibitor, eicosaenoid are oxygenated metabolites of arachidonic acid and interfered in signal transduction in insect immune responses. Among the important function ascribed to eicosaenoid is the central role they play as mediator in cellular

defense to bacterial infection in insect. They regulate bacterial clearances from the insect haemocoel. The inhibitor of arachidonic acid hydrolysis from cellular phospholipides by phospholipase A_2 PLA_2 as well as derivatives of arachidonic acid namely the products of the (COX) and (LOX) pathways decrease both bacterial clearances and nodulation in insect.

Insect biosynthesis verity of eicosaenoid in fat body and haemocytes eicosaenoid biosynthesis inhibitor inhibited the product of these compound. Garcia *et al.*, (2004) provided evidences that insect treated with eicosaenoid biosynthesis inhibitor become more susceptible to pathogenic infection and demonstrated that arachidonic acid metabolites are required for haemocytes microaggregation and activation of proPO system induced by the parasites. Thus supported the view that eicosaenoid modulated the immune response in *Rhodnius prolixus* infected with *Trypanosoma rangeli* infection. However, this eicosaenoid biosynthesis inhibitor could not be made use of during present investigation and hence no discussions on the utility of these in *H.armigera* can be made.

5.8 Total haemocyte counts

It is known that nucleo polyhedrosis virus multiply in haemocytes however the reports are scanty on changes of haemocytes number during the course of infection, therefore, the study on the total haemocyte count were undertaken and the results are presented in Table 19 and depicted in Fig.20 The results indicated that THC of both virosed and normal 3rd instar larvae of *H. armigera* were same at first day which later on, goes on increasing in normal larvae up to forth day after. Whereas, in case of virosed larvae, THC increased up to second day and decreased there after, continuously. However in 5th instar larvae of *H.armigera*,THC was found almost same in both normal and virosed larvae at first day after infection but thereafter, it declined consistently till the fourth day after infection. However, the rate of decline was more in virosed larvae as compared to normal.

Shapiro (1967) in the greater wax moth, *Galleria mellonella*, during the course of inoculation with Nucleopolyhedrosis and starvation, found no change in the total haemocytes count (THC) in inoculated larvae until day 10, when a significant decrease was observed as compared to 5th day. The THC's increased in control larvae, within the first 5 days of the test the THC's in control larvae was significantly greater than in inoculated larvae by the 5th day and the THC in inoculated larvae was significantly higher than in starved larvae.

Wittig (1968) also observed the THC's of granulosis-infected and control larvae of armyworms from 4 days after start of the test, a sharp drop in the THC's with the appearance of capsule in the blood, larvae fed with capsule suspension had mean THC of 20,215 haemocytes/mm^3, whereas, control larvae averaged 24,870 haemocytes/mm^3. This 19% decrease was significant at the 5% level. The number of haemocytes per unit volume of blood was greatly reduced during the latter part of the disease i.e. in moribund larvae.

Similarly, Shapiro *et al.*, (1969) examined haemocyte changes in larvae of the bollworm *Heliothis zea* infected with a Nucleopolyhedrosis virus. The average number of circulating haemocytes in both virus-exposed and nonexposed bollworm increased significantly during the first day of exposure. After 3 days of exposure, the average number of haemocytes decreased dramatically in larvae exposed to high virus dose. A similar decrease was not observed in nonexposed larvae or larvae exposed to a low virus dose. During present investigation both the 3rd and 5th instar larvae were exposed to their LC_{50} doses, respectively and hence the increase in haemocytes in 5th instar larvae over 3rd was related with age of larvae.

Jacob and Subramaniam (1974) reported identical results in Tobacco caterpillar *Spodoptera litura* F., infected with nuclear-polyhedrosis virus. The number of circulating haemocytes (THC) was significantly higher in infected larvae at 24 hour after inoculation. But at 48,72,96, and 120 hour following inoculation, the THC was significantly lower than in the corresponding healthy larvae. Further, the THC decreased progressively from 48 hour onwards in infected larvae as against a general increasing trend in healthy ones. The same was true for the 3rd instar normal larvae of *H. armigera,* whereas, both 3rd and 5th instar virosed larvae have decreased THC.

Like wise Rabindra and Subramaniam (1974) reported steady decrease in average number of haemocytes in *H. armigera* infected larvae with nuclear polyhedrosis virus and decrease was drastic after 48 hours from 28640/mm^3 to 15085/mm^3 at 120 hours.

Lea (1986) also found that in Wax Moth, *Galleria mellonella* infected with a virus *Sericesthis* Iridescent, the Total haemocyte counts were generally lower in infected insect than in control group. The relative depression of total count in infected group is especially pronounced in the day 12 sample in which the average total count of the infected group was

about 50% that of the control group. However, they have reported an increase in THC as larvae approach to pupation, which was so in 3rd instar but was not observed in 5th instar

Similarly, Kalia *et al.*, (2001) reported that the total haemocytes counts (THC) in *H. armigera* haemolymph were affected by nucleopolyhedrovirus (NPV) treatment. There was a general decrease in THC in response to NPV treatment in both young and old larvae. However the decrease was more apparent in 5 and 8 day old larvae than in 10-day-old larvae.

The reduction in THC in virosed larvae might be due to the invasion of the nuclei of blood cells by the virus, as polyhedral bodies could be seen in the nuclei of blood cell by 72 hours post-inoculation. As found by Rabindra and Subramaniam, (1974) and Kalia *et al.*, (2001) reported that when a polyhedral suspension was injected into the 10day old larvae the Plasmatocytes and Granular cells phgocytosed almost all polyhedra within 15 to 30 min of injection. But after 3 to 4 hr the immunocytes become vacuolated and disintegrated cells.

Bidochka *et al.*, (1989) suggested that in Oedipodinae grasshopper, PO might be associated with the haemocyte or other particulate material. The lower THC in virosed larvae is also correlated with the higher PO activity in plasma over that of cell fraction especially in 5th instar virosed larvae of *H. armigera, which* suggested disintegration of haemocytes and release of PO in to plasma. Kalia *et al.*,(2001) also reported the higher PO activity in plasma over that of cell fraction especially for day 9 of larval age and also suggested disintegration of haemocytes and release of PO in plasma. This correlated with the decrease in THC counts before pupation.

From the above discussion, it originate that we were not able to increase the efficacy of HaNPV to much extent due to use of PO inhibitor when compared with immuno suppressant like eicosaenoid biosynthesis inhibitor which made the *Rhodnius prolixus* more susceptible to pathogen. For the first time, the characterizations of haemolymph PO was done in *H. armigera,* these studies will go as foundation for further studies to understand the 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 that is concerned with immunity in insect. The NPV are highly species specific but specificity does vary and capacity for and frequency of change needs to be closely watched as change may present a major hurdle in the use of these. Hence the need for search of appropriate immuno suppressant presently hold good scope for their further utilization in insect pest management.

CHAPTER VI

SUMMARY

Helicoverpa armigera (Hubner) Hardwick is the most dreaded pest of almost all the cultivated crops in India. It causes massive damage to many important crops. However, exclusive remains on chemical insecticides and their injudicious use lead to the problems like resistance. To prevail over this dilemma use of biological control agent, in particular the baculoviruses having species specificity can help to reduce an elite reliance on chemical insecticides. But as the age of the larvae increases there is decrease in susceptibility of the larvae to HaNPV which is attributed to increase in biomass, less time for incubation as larvae pupate, more over pre gut and post gut barriers which include Phenoloxidase system. In view of this the work on study of phenoloxidase system and melanization in *H. armigera* (Hubner) exposed to HaNPV was under taken in present study.

Present work was performed during 2003-2005 in the Department of Entomology and Insect Biotechnology laboratory Dr. PDKV, Akola with aims, to conduct bioassay of HaNPV for different larvae instar of *H. armigera*, role of phenoloxidase cascade as a component of internal defenses against HaNPV in relation to age related resistance, to find out any correlation between colure of larvae, toxicity to HaNPV and PO level in the filed collected population of *H. armigera*, in vitro inhibition of PO and to study the synergist effect of different PO inhibition in combinations with HaNPV in laboratory and lastly the total hemocyte count. The results are summarized below.

6.1 Age related bioassay of *H.armigera* with HaNPV

The LC_{50} values for second, third, fourth and fifth instar larvae were 2.1 x 10^3, 1.0 x 10^3, 3.4 x 10^5 and 2 x10^6 POBs/ml, respectively. The second instar larvae was found to be 4.91, 161.73, 690.57 time more susceptible as compared to third, fourth and fifth instar larvae of *H. armigera*, respectively. The third instar larvae were 32.97 and 195.83 times more susceptible than fourth and fifth instar larvae respectively, whereas, the fourth instar larvae were 15.9 times more susceptible than fifth instar larvae.

6.2 Determination of phenoloxidase in different age groups of *H. armigera*

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

6.3 Characterization of phenoloxidase from normal and virosed larval haemolymph of *H. armigera*

6.3.1 Kinetics of phenoloxidase

When L-tyrosine was used as a substrate Km values of PO for normal and virosed were 1.36mM and 1.21mM respectively. Likewise Vmax values for normal and virosed larvae were 0.333 and 0.377ΔA/mg/min. In case of L-Dopa as a substrate, the Km values of 2.85 mM and 1.25mM were recorded, respectively for normal and virosed larvae. Similarly, Vmax values were 0.667 ΔA/mg/min and 0.832 ΔA/mg/min, for normal and virosed larvae, respectively. When Methylcatechol was used as a substrate, Km values were found 5.40 mM and 1.66 mM for normal and virosed haemolymph. However, Vmax values were 0.454 ΔA/mg/min and 0.667 ΔA/mg/min respectively. With Dopamine when used as a substrate, Km values of 6.23 mM and 5.55 mM and Vmax values of 1.053 ΔA/mg/min and 1.587 ΔA/mg/min, respectively were recorded for normal and virosed larvae. For DL-Dopa as a substrate, Km values were 3.03mM and 2.00mM and Vmax 0.571 ΔA/mg/min and 0.832 ΔA/mg/min, respectively for normal and virosed larvae. So in general in all the substrates studied, Km values decreased and Vmax values increased in virosed as compared to normal larvae of *H. armigera.*

6.3.2 Substrate Specificity

Among the substrates tested, Dopamine was found to be the best as it recorded maximum phenoloxidase activity in both normal and virosed *H. armigera* larvae. And also recorded maximum percentages increase in virosed as compared to normal haemolymph, which is followed by L-Dopa, DL-Dopa, Methylcatechol and L-Tyrosine. The study revealed

dopamine as the best substrate and is recommended to assess the PO activity in *H.armigera* as against Dopa, which is oftenly used in most of the PO study.

6.3.3 Optimum Temperature

An increase in enzyme activity was observed with increase in the temperature up to 40^{0}C and maximum PO activity was recorded at 40 °C (1448.11) 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 °C. The PO activity both from normal and virosed haemolymph decreased after 50^{0}C and reached to minimum of 5.90 Unit/ mg protein/min 80^{0}C 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.

6.3.4 Temperature stability

PO stability at different temperatures indicated that the PO from normal larvae of *H. armigera* showed almost 100 % activity at 10 °C, 20 °C and 30°C up to 80 min, thereafter, 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 °C up to 80 min interval. The enzyme activity was reduced afterwards.

6.3.5 Optimum pH

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. The optimum pH for PO activity was 9.0. It was also observed that PO was more active in alkaline pH as compared to acidic pH.

6.4 Phenoloxidase activity and HaNPV toxicity in different chromotonal *H. armigera* larvae

No particular trend was observed in LC_{50} values and PO activity in relation to colouration. It revealed that there was no relation in colour of larvae, HaNPV toxicity and phenoloxidase activity.

6.5 Inhibitor Specificity

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.

6.6 Electrophoretic study

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

Whereas haemolymph of virosed *H. armigera* also possess two PO isozymes (POV1 and POV2). POV1 possess Rf value approximately similar to PON1 and PON2 suggesting that it possess more or less same molecular weight and therefore it can be inactive Prophenoloxidase. Unlike POV1, POV2 possess very small Rf value and its smaller molecular weight is suggestive of activated phenoloxidase obtained after the cleavage of prophenoloxidse.

6.7 Effect of different phenoloxidase inhibitors as synergist with HaNPV on *H.armigera*

There was no much difference observed in LC_{50} values of HaNPV with inhibitor incorporated and in devoid of it. Same effect was noted in mortality of larvae however the intensity of effect was very little.

6.8 Total haemocyte count

Total haemocyte count (THC) of both virosed and normal 3rd instar larvae of *H. armigera* were same at first day which later on, goes on increasing in normal larvae up to forth day. Whereas, in case of virosed larvae, THC increased up to second day and decreased there after, continuously. However in 5th instar larvae of *H.armigera* THC was found almost same in both normal and virosed larvae at first day after infection but thereafter, it declined consistently till the fourth day after infection. However, the rate of decline was more in virosed larvae as compared to normal.

CONCLUSIONS

From the study of phenoloxidase and melanization in *H.armigera,* when exposed to HaNPV, conducted for two consecutive years in the laboratory, following conclusions were drawn.

1) The mortality of *H.armigera* due to HaNPV is age maturation phenomenon; as age of larvae increases, the susceptibility decreases.
2) Phenoloxidase activity in 3rd and 5th instar larvae of *H. armigera* was more in cellular fraction in normal whereas it was high in plasma in virosed 3rd and 5th instar. However, its magnitude was more in virosed larvae of 5th instar as compared to 3rd instar, which is a sign of more rapid vigorous melanization and encapsulation reaction in response to HaNPV exposure.
3) The Km of PO from virosed larvae decreased as compared to normal larvae which means that the affinity of enzyme towards substrate increases in virosed larvae. This indicated that most of the PO is free or activated due the infection of HaNPV, due to which the affinity of all tested substrate towards enzyme increases.
4) The Vmax in virosed larvae increased over that of normal larvae indicating that amount of PO was more in virosed larvae as compared to normal. These higher levels of PO in haemolymph could lead to a more rapid, vigorous melanization and encapsulation reaction in response to exposure to HaNPV infection.

5) Among the substrates tested Dopamine was found to be the best and is recommended for insect PO study instead of more oftenly used Dopa.
6) The optimum temperature for PO enzyme activity in both normal and virosed larvae was 40^0C.
7) The PO from both normal and virosed larvae of *H. armigera* showed almost 100 % enzyme activity up to 30^oC; which declined thereafter with rise in temperatures.
8) The optimum pH for PO activity was found to be 9.0 for both normal and virosed *H.armigera* larvae.
9) No correlation was observed in colour of larvae, HaNPV toxicity and phenoloxidase activity in *H.armigera.*
10) Amongst all the inhibitors studied, SDS was found to be the best inhibitor of haemolymph PO followed by EGTA >PTU> EDTA > PMSF> DMSO > Garlic extract.
11) In both the normal and virosed larval haemolymph of *H armigera,* two PO isozymes were present. In normal haemolymph, both isozymes migrated in close proximity that can be inactive Prophenoloxidase. Unlike normal, virosed haemolymph posses one band of higher Rf and other small band of very small Rf value suggesting it as activated phenoloxidase, which cleavaged from prophenoloxidse.
12) Due to incorporation of the inhibitors with HaNPV, very little effect was noted in mortality of larvae
13) At first day after infection, THC of both virosed and normal 3rd instar larvae remain same. In case of virosed larvae, THC increased up to second day, but there after, it declined continuously, whereas, THC increased in normal larvae up to fourth day.
14) In both normal and virosed 5th instar larvae, THC was almost same at first day after infection but thereafter, it declined consistently till the fourth day after infection. However, the rate of decline was more in virosed larvae as compared to normal
15) The lower THC in virosed larvae is also correlated with high PO titer in plasma fraction of 5th instar larvae. This suggested disintegration of haemocytes and release of PO in plasma.

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* Originals not seen

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THESIS ABSTRACT

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b) Full name of the student	: Sadawarte Ajay Krishnakumar
c) Name and address of major Advisor	: Shri M.N.Nachane, Assoc Prof and Head, Department of Entomology, Dr. PDKV, Akola
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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 and also measures for increasing the efficacy of HaNPV. The studies were undertaken in the Department of Entomology and Insect Biotechnology laboratory of Dr.PDKV, Akola during 2003-05. The work on bioassay of HaNPV for different larval instars, role of phenoloxidase (PO) in age related resistance, characterization of PO, correlation of colour of larvae with toxicity and PO level, effect of

inhibitors on larval mortality when feed with HaNPV and difference in total haemocyte count in normal and virosed larvae was also done. The results in brief are presented below. The LC_{50} values for second, third, fourth and fifth instar larvae were 2.1 x 10^3, 1.0 x 10^4, 3.4 x 10^5 and 2 x10^6 POBs/ml, respectively. Susceptibility was found to decrease as the age of larvae increased.

Phenoloxidase activity was more in cellular fraction of haemolymph 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 indicating that the affinity of enzyme towards substrate increases in virosed larvae whereas the Vmax in virosed larvae increased over that of normal larvae which indicated that amount of PO is more in virosed larvae due to HaNPV infection. Dopamine was best substrate than any other studied and is recommended for insect PO study. Enzyme PO was optically active at 40^0C in normal larvae as well as virosed larvae. The PO from normal and virosed larvae of *H. armigera* showed almost 100 % enzyme activity up to 30^oC, which decreased thereafter. The optimum pH for PO activity was found to be 9.0 for normal and virosed *H.armigera* larvae.

No relation in colour of larvae, HaNPV toxicity and phenoloxidase activity was observed. 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 haemolymph of normal and virosed *H armigera* larvae. In normal larvae both isozymes migrate in close proximity and are of prophenoloxidse. Unlike normal, virosed posses one band of higher Rf, which is of prophenoloxidse, and other small band of very small Rf value, which is of phenoloxidase. Very little effect was noted in mortality of larvae due to incorporation of the inhibitors with HaNPV. There was decrease in THC due to HaNPV infection in virosed larvae as compared to normal and this decrease correlated with increase in PO activity in plasma of virosed larvae.