BIOCHEMICAL STUDIES ON MITOCHONDRIA AND STORAGE PROTEINS OF *HELICOVERPA ARMIGERA* (Hubner)

Thesis submitted to
Gulbarga University, Gulbarga, for
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Doctor of Philosophy
in

BIOCHEMISTRY

By

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This thesis is dedicated to my beloved parents and Family members for their love, endless support and encouragement.

DECLARATION

I hereby declare that the matter embodied in this thesis entitled, **"Biochemical Studies on the Mitochondria and Storage Proteins of *Helicoverpa armigera* (Hubner)"** submitted to Gulbarga University, Gulbarga for the award of the degree of **Doctor of Philosophy** in **BIOCHEMISTRY** is the result of investigations carried out by me in the Department of Biochemistry under the supervision of **Dr K. Sreeramulu** and it has not been submitted previously in part or in full, for the award of any degree/ diploma of this or any other University/Institution.

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CERTIFICATE

This is to certify that the thesis entitled, ***"Biochemical Studies on Mitochondria and Storage Proteins of Helicoverpa armigera (Hubner)",*** *submitted by* ***Mr. S MD Akbar*** *for the award of the degree, Doctor of Philosophy in* ***BIOCHEMISTRY*** *to Gulbarga University, Gulbarga, is a original research work carried out by him under my guidance. The thesis or part thereof has not been previously submitted any where for any other degree.*

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S MD Akbar

Contents

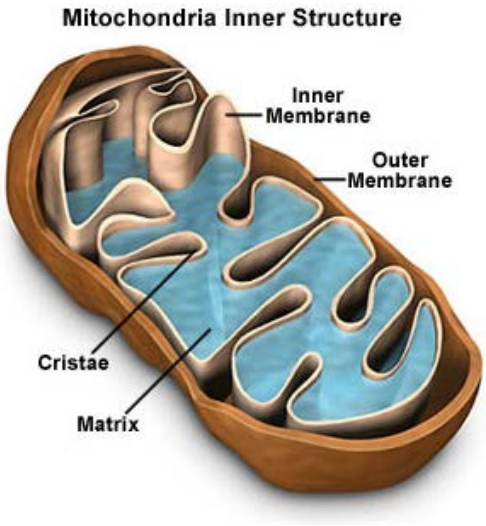
LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS:

Ha	*Helicoverpa armigera*
ATP	Adenosine triphosphate
ADP	Adenine diphosphate
AMP	Adenine monophosphate
TMR	Tetramethyl rosamine
PVDF	polyvinyl difluoride
LC-MS/MS	Liquid chromatography / Mass Spectroscopy
Trp	Tryptophan
μg	Microgram
μl	Microliter
ml	Milliliter
μM	Micromole
mM	Milimolar
mg	Milligram
M	Molarity
w/v	Weight per volume
w/w	Weight per weight
nm	Nanometer
°C	Degree Celsius
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium chloride
$MgCl_2$	Magnesium chloride
$NaCl_2$	Sodium chloride
KCl_2	Potassium chloride
$FeCl_3$	Ferric chloride
$HgCl_2$	Mercurous chloride
TCA	Tetra chloro acetic acid
H_2O_2	Hydrogen peroxide

HCl	Hydrochloric acid
DMSO	Dimethyl sulphaoxide
DTT	Dithiothretal
EGTA	Ethylene glycol tetra acetic acid
EDTA	Ethylene tetra acetic acid
PTU	Phenyl thiourea
***P*-CMBS**	p-chloro mercuri benzene sulfonate
PMSF	Phenylmethylsulfonyl fluoride
CHAPS	3[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid;
SDS	Sodium dodecyl sulphate
et al.,	And others
kDa	Kilo Dalton
K_m	Michealis-Menton constant
U	Units
h	Hours
pH	Hydrogen ion concentration
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
V_{max}	Maximum velocity
HTP	Hydroxylappetite
TLC	Thin layer chromatography
LD	Lethal dose
Tris	Tris[hydroxymethyl]aminomethane
ROS	Reactive Oxygen species
SA	Salicylic acid
JA	Jasmonic acid

Chapter I

Introduction



1.1. *Helicoverpa*-The Global Problem

The legume pod borer or cotton bollworm, *Helicoverpa armigera* (Hubner), is one of the most important constraints to crop production globally. It is polyphagous and attacks more than 182 plant species, including cotton, chickpea, pigeonpea, peas, cowpea, sunflower, sorghum, groundnut, field beans, tomato, tobacco, maize and a range of vegetables, fruit crops and tree species. *Helicoverpa armigera* is widely distributed in Asia, Africa, Australia and the Mediterranean Europe. Crop production in many countries, especially in the semi-arid tropics (SAT), is severely threatened by the increasing difficulty in controlling insect pests such as *H. armigera* (Sharma, 2005).

The food demand in the developing countries is likely to double by 2025 from the present level of production. Productivity of most cereals, pulses, and oilseeds in Southeast Asia continues to be low. For example, the productivity of rice in most countries in the Indian subcontinent is close to 2 tonnes ha^{-1}, whereas the average world productivity of rice is about 2.5 tonnes ha^{-1}. An almost similar trend is observed in most other crops. Among various biotic and abiotic factors responsible for low productivity, the damage from insect pests and diseases accounts for major losses in different crops. Among insect pests, *Helicoverpa armigera* (Hubner) is regarded as the most serious pest, which has a wide host range among the cultivated crops such as cotton, legumes, cereals, vegetables and fruits. In the South Asian region, *Helicoverpa* is a serious pest of cotton, chickpea, pigeon pea, groundnut, cowpea, *Vigna* species, okra, tomato, castor, sunflower, maize, sorghum and many more crops (Sharma, 2005). The extent of damage may vary from crop to crop and season to season. In extreme cases of infestation, the entire harvestable yield can be destroyed by this insect.

H. armigera is able to adapt to various cropping systems: high polyphagy, wide geographical range, mobility, migratory potential, facultative diapause, high fecundity and propensity to develop resistance to insecticides are physiological, ethological and ecological factors that have strongly contributed to its pest status (Fitt, 1989; Forrester et al., 1993; McCaffery, 1998).

1.1.1. Biology and Ecology

The life cycle of *H. armigera* is completed in 35-40 days at 25 ˚C. There may be up to 10 to 11 generations in a year. All stages of the insect are found throughout the year provided food is plenteous, but development is slowed or stopped by either drought or cold. The white yellow, ribbed and spherical eggs are laid in the early morning hours, which become dark black before hatching. Eggs are laid individually, preferably on or near the fruiting bodies or tender plant parts. Egg hatches in 2 to 4 days. The larvae vary in color, from green, brown and red to back, and have distinct longitudinal stripes running down the body. Larval integument is covered by numerous spines. The larvae usually feed on tender plant parts, flowers and fruits. There are five to six larval instars, and the larval development is completed in 12-16 days. As the larvae develop, the tendency for cannibalism increases. Fully developed larvae move into the soil where they pupate 5-15 cm below the soil surface in earthen cells. The red-brown pupae are nearly 2.5 cm long and pupal stage lasts for 10-12 days. Adults are dark, reddish brown, to olive green; their wings are pale, bordered by a dark band. Females lay upto 300-700 eggs. Adults are nocturnal in habit and emerge in early hours of day (Sharma, 2005).

1.1.2. *Helicoverpa* management

Helicoverpa pose as major pest on several crops, and their pest status is rooted in their mobility, polyphagy, facultative diapauses as pupae, rapid generation turnover, fecundity and predilection for harvestable parts of high-value crops such as cotton, tomato, pulses and cereals. The high level of control required under these circumstances means that chemical or best integrated control methods need to be adopted to minimize the losses due to these pests. Pest management strategies for *Helicoverpa* include, cultural manipulations of the crop and its environment; host plant resistance, including wide hybridization and transgenics; biological control, including the use of microbial pesticides; sex pheromones for the population monitoring or mating disruption; and chemical control.

1.2. Insect-plant interactions

Insects make up the most diverse and abundant group of plant consumers. A total of 45% of the approximately 1 million described insect species feeding on plants (Schoonhoven et al., 2005). Given that the estimated number of insects species is several times higher (Stork, 2007), the number of herbivorous insects species is likely to be much higher too. Herbivorous insects may attack plants below ground as well as above ground, and not a single organ remains free of potential insect attack (Schoonhoven et al., 2005). Plants have evolved a range of defenses to ward off this diversity of attackers, including constitutive and induced defenses (Schoonhoven et al., 2005; Kessler and Baldwin, 2002). Because an individual plant may potentially be under the attack of tens of hundreds of consumer species, it is impossible to have constitutive defenses against all these attackers. Furthermore, whether the potential enemies will indeed attack a certain

individual is usually unpredictable. Moreover, constitutive defense may also select for adaption in herbivorous insects (Agarwal and Karban, 1999). Thus, inducible defenses may tune the defensive needs to the actual presence of attackers and in addition may transform plants into moving fortresses that have a modified phenotype and consequently retard adaptation in herbivores (Agarwal and Karban, 1999).

Induced defenses comprise direct defenses, such as secondary metabolites and protease inhibitors that negatively affect herbivore growth and survival, as well as indirect defenses, such as herbivore-induced plant volatiles and herbivore-induced extrafloral nectar that enhances the effectiveness of natural enemies of herbivores, such as parasitoids or predators (Kessler and Baldwin, 2002; Kappers et al., 2005). The induction of defenses is often specific for the attacker species (Kahl et al., 2000; Arimura et al., 2005) and an individual plant can therefore express a range of different phenotypes where each phenotype has its own effects on the members of the community, such as herbivores, carnivores, and pollinators (Kessler et al., 2004; Kessler and Halitschke, 2007). Salicylic acid (SA), jasmonic acid (JA), nitric oxide (NO) and reactive oxygen species (ROS) (particularly H_2O_2) increase in abundance following pathogen recognition and each are important signaling molecules that promote and coordinate defense and HR responses (Goggin, 2007; Sasan et al., 2007).

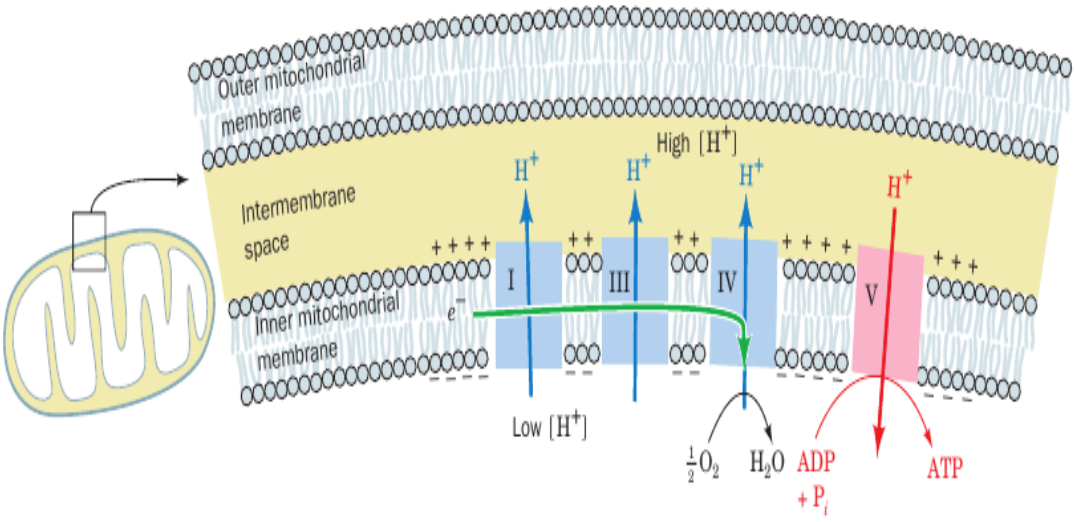
Because an individual plant may potentially be under the attack of hundred of insect species, it is impossible to depend on plants' natural defense mechanism for the control of agricultural insects. So, man had started applying synthetic insecticides for their control. Insecticides exhibit a high level of pest control ability combined with a relatively low degree of environmental toxicity. Almost all classes of insecticides are

neurotoxic in nature, hence, they are used widely around the world in agriculture and in households. There are a number of reports on the mode of action of insecticides on insects as well as on non-target groups with different target sites (Ellison et al., 2011; Rai et al., 2009).

1.3. Oxidative phosphorylation and oxidative stress

Mitochondria generate cellular energy in the form of ATP (adenosine triphosphate) by the process of oxidative phosphorylation. Most cells contain hundreds of mitochondria. These cytoplasmic organelles are thought to have arisen about 1.5 billion years ago from a symbiotic association between a glyolytic proto-eukaryotic cell and an oxidative bacterium. Oxidative phosphorylation is a metabolic pathway that uses energy released by the oxidation of nutrients to produce adenosine triphosphate (ATP). Although the many forms of life on earth use a range of different nutrients, almost all carry out oxidative phosphorylation to produce ATP, the molecule that supplies energy to metabolism. This pathway is probably so pervasive because it is a highly efficient way of releasing energy, compared to alternative fermentation processes such as anaerobic glycolysis.

In mitochondria, oxidative phosphorylation results from the coupling between the redox-primary proton pumps in the respiratory chain and the F_1F_0-ATP synthase. The proteins involved in oxidative phosphorylation are located within the mitochondrial inner membrane and include the electron transport chain (ETC) components, ATP synthase, and the adenine nucleotide translocator (ANT) (Fig. 1.1). The ETC oxidizes hydrogen derived from the oxidation of organic acids such as pyruvate and fatty acids with atomic oxygen to generate water. The electrons, borne on NAD^+ (nicotinamide adenine



(Source: Voet and Voet, 2011, Biochemistry, 4th Ed.)

Fig. 1.1. Mitochondrial inner membrane showing electron transport chain.

dinucleotide), are transferred to respiratory complex I (NADH dehydrogenase) and then to coenzyme Q10 (CoQ), and the electrons from succinate in the tricarboxylic acid (TCA) cycle are transferred to complex II (succinate dehydrogenase, SDH) and to CoQ. From CoQ, the electrons are passed to complex III, then to cytochrome *c* (cyt *c*), then to complex IV (cytochrome *c* oxidase, COX), and finally to oxygen to give water. The energy released is used to pump protons (H^+) out of the mitochondrial inner membrane to create an electrochemical gradient that is positive and acidic on the outside and negative and alkaline on the mitochondrial matrix side. This creates a capacitor that can be depolarized by the transport of protons back into the matrix through a proton channel in the F_0 membrane component of the ATP synthase. The proton flux drives the condensation of ADP (adenosine diphosphate) and Pi (inorganic phosphate) to make ATP, which is then exported to the cytosol in exchange for the spent ADP by the ANT. In this way, oxygen consumption by the ETC is coupled to ADP phosphorylation by the ATP synthase through the electrochemical gradient.

Oxidative stress (OS) can be defined in terms of a shift of the balance between prooxidants and total antioxidants in the cells towards the former. The ROS and free radicals (FR) have the ability to cause peroxidation of unsaturated lipids constituting the membrane of cells and depletion of cellular reserves of reducing elements (both enzymatic and nonenzymatic; jointly called as antioxidants defense system), which the body can produce indigenously. The FR are the chemical species, that are defined in terms of the atoms, or molecules which contain one or more unpaired electrons and that makes them several folds more reactive than their corresponding ions and FR continue to seek stabilization by reacting with other micromolecules, failing which they may interact

with biological macromolecules or tissues. FRs have various chemical forms such as hydroxyl, superoxide, nitric oxide and lipid peroxyl radicals etc. The production of FR has been used in the genesis of toxicity of many man-made chemicals and drugs that are called as xenobiotics.

The aerobic organisms including human beings utilize molecular oxygen for respiration and oxidation of nutrients through mitochondria, a powerhouse of the cell, as it generates energy for driving its physiological activities. As a consequence of the mitochondrial activity, highly reactive oxygen species (ROS) are generated. ROS comprise FR, H_2O_2 and peroxynitrite anion. The FR elements are highly unstable due to available electrons; they readily react with various cellular organic substrates such as lipids, proteins, membranes and DNA. Oxidation of these molecules can result into molecular damage which further leads to alterations in their normal biochemical/physiological functions. As a consequence of oxidation of nutrients in mitochondria and production of ATP through ETC, FRs are frequently generated. The main source of ROS *in vivo* is aerobic respiration, although ROS are also produced by peroxisomal β-oxidation of fatty acids, microsomal cytochrome P450 metabolism of xenobiotic compounds, stimulation of phagocytosis by pathogens or lipopolysaccharides, arginine metabolism, and tissue specific enzymes.

Oxidative phosphorylation is the major endogenous source of the ROS (O_2^-, H_2O_2, and $OH^·$), which are toxic by-products of respiration. This is because when the ETC is inhibited, the electrons accumulate in the early stages of the ETC (complex I and CoQ), where they can be donated directly to molecular oxygen to give superoxide anion (O_2^-). Superoxide anion is detoxified by the mitochondrial Mn superoxide dismutase (MnSOD)

to give H_2O_2, and H_2O_2 is converted to H_2O by catalase. H_2O_2, in the presence of reduced transition metals, can also be converted to the highly reactive hydroxyl radical ($OH^{\cdot}$) by the Fenton reaction. Chronic ROS exposure can result in oxidative damage to mitochondrial and cellular proteins, lipids, and nucleic acids, and acute ROS exposure can inactivate the ironsulfur (Fe-S) centers of ETC complexes I, II, and III, and TCA cycle aconitase, resulting in shutdown of mitochondrial energy production (Wallace, 1999).

Mitochondria also provide a major switch for the initiation of apoptosis. This switch is thought to involve the opening of a nonspecific mitochondrial inner membrane channel, the mitochondrial permeability transition pore (MPTP). The mitochondrial inner membrane space contains a number of cell death-promoting factors, including cyt *c*, apoptosis-inducing factor (AIF, a flavoprotein), and latent forms of specialized proteases called caspases. Opening of the MPTP causes collapse of electrochemical gradient, swelling of the mitochondrial inner membrane, and release of these death-promoting factors. The cyt *c* activates the cytosolic caspase protein degradation pathway, leading to destruction of the cytoplasm. AIF translocates to the nucleus, inducing chromatin destruction (Wallace, 1999). Opening of the MPTP and the accompanying death of the cell can be initiated by the mitochondrion's excessive uptake of Ca^{2+}, increased exposure to ROS, or decline in energetic capacity (Wallace, 1999). Thus, a marked reduction in mitochondrial energy production and a chronic increase in oxidative stress could theoretically activate the MPTP and initiate apoptosis. The interaction of these three oxidative phosphorylation processes may explain some of the perplexing pathophysiological features of mitochondrial disease. For example, mutations that disrupt

mitochondrial oxidative phosphorylation would reduce energy output and presumably impair multiple cellular processes. Inhibition of the ETC is likely to increase ROS production and oxidative stress. Increased oxidative stress and decreased energy levels might activate the MPTP, leading to apoptosis.

1.4. Synthetic pesticides

A pesticide is any chemical which is used by man to control pests. The pests may be insects, plant diseases, fungi, weeds, nematodes, snails, slugs, etc. Therefore, insecticides, fungicides, herbicides, etc., are all types of pesticides. Some pesticides must only contact (touch) the pest to be deadly. Others must be swallowed to be effective. The way that each pesticide attacks a pest suggests the best way to apply it; to reach and expose all the pests. For example, a pesticide may be more effective and less costly as a bait, rather than as a surface spray. An insecticide is a pesticide used against insects in all developmental forms. They include ovicides and larvicides used against the eggs and larvae of insects. Insecticides are used in agriculture, medicine, industry and the household. Insecticides act by poisoning the nervous system of target organisms - including man if dose is sufficiently high. The use of insecticides is believed to be one of the major factors behind the increase in agricultural productivity in the 20th century. Nearly all insecticides have the potential to significantly alter ecosystems; many are toxic to humans; and others are concentrated in the food chain. It is necessary to balance agricultural needs with environmental and health issues when using insecticides. Some examples of chemically-related pesticides follow:

Organophosphate Pesticides - These pesticides affect the nervous system by disrupting the enzyme that regulates acetylcholine, a neurotransmitter. Most organophosphates are

insecticides. They were developed during the early 19th century, but their effects on insects, which are similar to their effects on humans, were discovered in 1932. Some are very poisonous (they were used in World War II as nerve agents). However, they usually are not persistent in the environment.

Carbamate Pesticides affect the nervous system by disrupting an enzyme that regulates acetylcholine, a neurotransmitter. The enzyme effects are usually reversible. There are several subgroups within the carbamates.

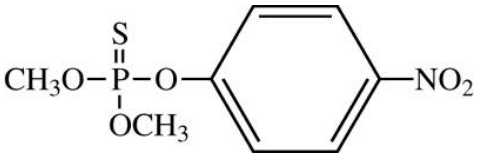
Organochlorine Pesticides were commonly used in the past, but many have been removed from the market due to their health and environmental effects and their persistence (e.g. DDT and chlordane).

Pyrethroid Pesticides were developed as a synthetic version of the naturally occurring pesticide pyrethrin, which is found in chrysanthemums. They have been modified to increase their stability in the environment. Some synthetic pyrethroids are toxic to the nervous system.

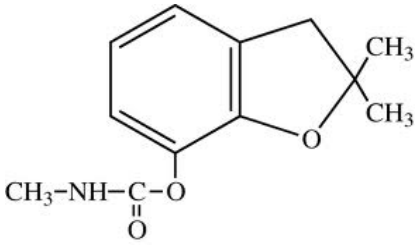
1.4.1. Insecticides and the mitochondria

During the last years, mitochondrial damage has been recognized as a cause for side effects of many drugs and toxins including insecticides. Mitochondria representing a target for toxins' toxicity are not surprising, since they have a central function in cellular energy production and contain multiple metabolic pathways. The respiratory chain and β-oxidation of fatty acids, are frequent targets of mitochondrial toxins. A list of identified mitochondrial toxins according to their principle mechanism is given in Table 1.1.

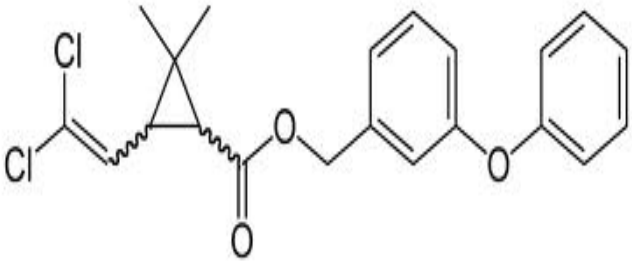
Several insecticides have been shown to affect the mitochondrial bioenergetics by a variety of mechanisms. One well-defined mechanism is inhibition of the electron flow



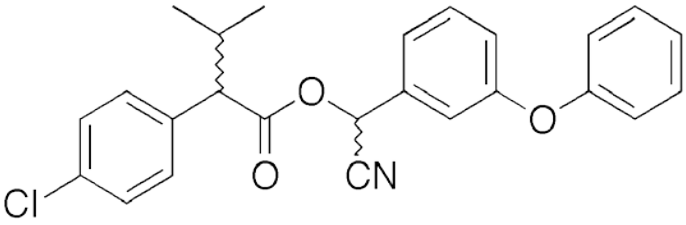
Methylparathion



Carbofuran



Permethrin



Fenvalarate

Fig. 1.2. Structures of the insecticides.

Table 1.1: Mitochondrial toxicity of drugs: principle mechanisms and typical examples (Wallace, 1999)

Inhibition of the electron transport chain
Amiodarone, anthralin, buprenorphine, flutamide, MPP +, oxmetidine, perhexiline
Uncoupling of oxidative phosphorylation
Amiodarone, bupivacaine, buprenorphine, etidocaine, tacrine
Mitochondrial permeability transition
Salicylate, valproate
Inhibition of mitochondrial fatty acid metabolism
Amiodarone, buprenorphine, female sex hormones, NSAIDs, salicylate, tetracycline, valproate
Oxidation of mitochondrial DNA
Alcohol
Inhibition of mitochondrial DNA synthesis
Nucleoside analogues, e.g. zidovudine, fialuridine

across the ETC (enzyme complexes I-IV). Typical examples for such compounds are: carbofuran exposure to rats inhibited the mitochondrial respiratory functions which were assessed in terms of reduction of MTT (3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) and a decrease in the activity of succinate dehydrogenase (Kamboj et al., 2008), monocrotophos and dichlorvos inhibited the activities of respiratory complexes I, III and IV in isolated mitochondria from rats exposed to those insecticides (Masoud et al., 2009), etc. The molecular mechanisms by which these insecticides inhibit the electron flow across the ETC are in general not well established. Two possibilities, how these substances can impair electron flow in the ETC, are by direct inhibition of a protein subunit of one (or more) of the enzyme complexes or as an acceptor of electrons instead of ubiquinone and/or cyt *c*.

Uncoupling of oxidative phosphorylation is another well defined mechanism for mitochondrial toxicity. Uncoupling means that the flow of electrons across the ETC and

the shift of protons from the mitochondrial matrix to the intermembrane space function normally, but the protons to not pass across the F_0F_1ATPase (complex V) back to the mitochondrial matrix but directly across the inner mitochondrial membrane. The result is production of heat, but not of energy in the form of ATP. Typical examples for this mechanism include weak acids or bases such as salicylic acid and nonsteroidal anti-inflammatory drugs (Krahenbuhl, 2001). For the weak bases tacrine and amiodarone, the most likely mechanism of uncoupling is protonation to the quarternary amine in the intermembrane space, transport across the inner mitochondrial membrane by the membrane potential and deprotonation in the mitochondrial matrix where the pH is basic. The base can then diffuse back across the inner mitochondrial membrane into the intermembrane space and the cycle is closed. A similar mechanism can be postulated for weak acids. By this mechanism, protons are carried across the inner mitochondrial membrane without passing across the F_0F_1ATPase, avoiding the production of ATP.

Opening of a mega-channel in the inner mitochondrial membrane, leading to swelling of the mitochondria (permeability transition) and collapse of the membrane potential, is another potential mechanism for insecticide-toxicity. Well known stimuli for this permeability transition of the inner mitochondrial membrane include calcium, ischemia, oxygen radicals, agonists of the mitochondrial benzodiazepine receptor and others (Zoratti and Szabo, 1995). Opening of this mega-channel can lead to mitochondrial loss of cyt *c*, initiating the apoptosis cascade, eventually ending in cell death. Drugs which have been associated with this type of liver toxicity include salicylic acid and valproic acid (Trost and Lemasters, 1996). Several drugs have been shown to inhibit hepatic fatty acid metabolism, in particular mitochondrial β-oxidation of fatty acids.

Examples for such drugs include salicylate, valproate, tetracycline, nonsteroidal anti-inflammatory drugs, female sex hormones and amiodarone (Fromenty and Pessayre, 1995).

Mitochondrial metabolism is responsible not only for the generation of energy, but also for FRs (Beal, 1998). Thus, some studies suggest that the exposure to a wide range of pesticides could result in the production of FRs and the inactivation of components of the mitochondrial respiratory chain (Gassner et al., 1997; Turner et al., 2003). Mitochondria is the primary sub-cellular target of the organophosphorous compounds inhibited ibits the enzymatic activity and the ATP generation, causing structural alterations of the matrix and mitochondrial swelling (Tos-Luty et al., 2003). Almost all intracellular ATP is known to be generated in the mitochondria and about one-third of the cellular adenine nucleotides are located in this organelle (Papa, 1996); therefore, chemicals causing mitochondrial dysfunction may deplete ATP, leading to excessive generation of ROS (Tsujimoto, 1997).

A final mechanism is damage to the mitochondrial DNA by oxidation or by inhibition of DNA synthesis (Krahenbuhl, 2001). Mitochondrial DNA is more susceptible to oxidative damage than nuclear DNA due to absence of histones and efficient repair mechanisms in mitochondria, and also due to the proximity of mitochondrial DNA to the oxygen radicals produced by the respiratory chain.

1.4.2. Insecticides and the oxidative stress

An extensive survey of available literature indicates that for the last two decades insecticides-induced OS has been considered as a possible mechanism of toxicity. The insecticides have been shown to induce production of ROS by altering the balance

between the oxidants / prooxidants and antioxidants through promoting lipid peroxidation (LPO) and depleting the antioxidative cellular reserves (both the enzymatic and non enzymatic) leading to a condition of OS. The range of its impact spans from tissue injury, and aging through apoptosis, to onset of various known/unknown diseases.

Every organism protects itself from the damages caused to it by FRs/ROS employing various defense mechanisms. The cells primarily utilize its indigenous antioxidant defense system comprising FRs/ROS scavenging enzymes as the first line of defense and the sulfhydryl reserves (thiols), vitamins (A, C and E) and their precursors, and polyphenols as the second to combat ROS/FRs mediated toxicity to human health (Annuk et al., 2001; Asano and Matsui, 1999). An antioxidant is a molecule that is capable of slowing down or preventing the oxidation of other molecules by ROS/FRs or any other chemical events.

Amal et al., (2005) have studied the effect of acute organophosphorus toxicity on the biomarkers of oxidative stress and apoptosis. They conducted biochemical assays for monitoring activities of true and pseudo cholinesterase, catalase and caspase 3 as well as levels of glutathione, (GSH), and malonyldialdehyde (MDA) and found significant decrease in the levels of reduced glutathione and catalase and increase in the level of MDA in OP exposed patients over controls. They observed a significant linear negative correlation between MDA and cholinestrases and a significant positive correlation between cholinestrases and catalase, and reduced glutathione (GSH). Thus they concluded that organophosphate in the patients induced production of OS as evidenced by the increased level of MDA (end product of lipid peroxidation) and decreased level of antioxidants (reduced glutathione and catalase). MDA level is considered as an index of

the general peroxidative damage to different tissues (Melchiorri et al., 1996), GSH serves as a substrate in the glutathione peroxidase/ glutathione reductase system, with NADPH as a reductant, to detoxify lipid peroxidation. Depletion of GSH favours lipid peroxidation and consequently induces cell damage (Maellaro et al., 1990; Verma et al., 2007). Catalase is one of the cellular defense mechanisms against cytotoxic oxygen species (H_2O_2). A decrease in its activity can lead to excessive accumulation of ROS resulting in initiation and propagation of lipid peroxidation. The impaired glutathione redox status could result in induction of apoptosis, as the activity of caspase-3 is elevated (Maellaro et al., 1990). Also, the activity of cholinestrases correlated negatively with MDA levels and positively with glutathione and catalase levels in OP toxicity. Similar to these results, the observations recorded from different organs (liver, kidney, spleen, and brain) of rats exposed to sublethal concentrations of chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothionate, CPF) through intramuscular route for 3 days revealed decrease in the levels of reduced glutathione (GSH) and consequent increase in oxidized glutathione (GSSG) levels, resulting in a significant decrease in GSH/GSSG ratio in all these rat tissues tested.

The chlorpyrifos (a chlorinated organophosphate) has also been reported to induce OS into liver, kidney, brain and fetus of the pregnant rats (Zama et al., 2007). The effects of carbofuran (CF, another carbamate pesticide) has been observed on the erythrocytes of rats keeping in view that RBCs are prone to oxidative stress due to the presence of hemoglobin in its centre and polyunsaturated fatty acids in its membrane lipids (Rai et al., 2009). Oxidative stress (OS) is associated with increased osmotic fragility of erythrocytes. They showed drastic increase in the activities of CAT and SOD and

decrease in the GST activity in RBC´s membrane. Recently, chronic exposure to carbofuran via oral administration has been reported by Rai and Sharma (2007) to generate ROS in rat brain and liver by exposing the animals (intra peritoneal, ip) to different subacute concentrations. The results demonstrated that carbofuran treatment at the 3 concentrations tested caused significant increase in lipid peroxidation (LPO). The increased oxidative stress at the same pesticide concentrations significantly induced activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase in rat brain; these results demonstrated that i.p. administration of carbofuran accelerated oxidative stress in rat brain in a dose-dependent manner. Similar findings were reported by several other workers into different parts of rat brain and brain mitochondria of carbofuran treated rats (Kamboj et al., 2006; Kamboj et al., 2008).

Koner et al., (1998) have shown that treatment of experimental animals with subacute doses of organochlorines induced OS in rats by evaluating the impact of DDT and lindane exposure on lipid peroxidation and antioxidant mechanisms in rats. They found that the oral administration of DDT and lindane dependently increased thiobarbituric acid reactive substance (TBARS) levels in serum after 8 wk of treatment. The activity of SOD in red blood cells (RBCs) also got increased by these compounds in dose dependent manner.

In another study, endosulfan has been reported to induce OS in rats heart as there was significant rise in the activities of SOD, glutathione peroxidase (GPx) and catalase (CAT) which could be prevented by use of vitamin E as an antioxidant (Jalili et al., 2007). Lindane and malathion treatment have also been shown to cause immunotoxicity in cell culture by inducing OS. Some workers (Olgun and Misra, 2006; Zhao et al., 2009)

have demonstrated that acetofenate, an organochlorine insecticide treatment can cause macrophage apoptosis by inducing oxidative stress on mouse macrophage cell line RAW264.7.

Some available reports indicate that certain pyrethrins have the potential to generate OS in various key tissues of some mammalian systems as reviewed by Abdollahi et al., (2004). Exposure of deltamethrin has been shown to induce OS and cause perturbations in various biochemical parameters including LPO, antioxidant and neutrotransmission enzymes; the toxicity however, has been shown to be reduced by treatment with vitamin E (Yousef et al., 2007). In rats brain and liver, cypermethrin induced OS has been observed which got ameliorated by treatment with vitamin E or allopurinol (Giray et al., 2001).

Biochemical parameters such as lipid peroxidation, lactate dehydrogenase, activities of antioxidant enzymes, etc., are measured for the evaluation of toxicity of pesticides (Ezeji et al., 2011). Several pesticides have been shown to stimulate peroxidation of cellular membranes (Dikshith, 1991) including: organophophorus insecticides [nitroaromatic complex (parathion, methyl-parathion, paraoxon), vinyl-derivatives (phosphamidon, mevinphos, temivenphos), O,Odimethylmalathion, malathion, trichlorfon, sumithion], organohalogenated pesticides [halogenated alkanes (carbon tetrachloride), tetrachlorodibenzodioxin, pentachlorophenol, hexachlorobenzene, dichlorodiphenyltrichloroethane, polychlorinated biphenyls, lindane, monuron, diuron], and herbicides (aminotriazole, paraquat). Pesticides and related compounds may contribute to the process of membrane peroxidation, by several mechanisms (Datta et al., 1994; Dikshith, 1991; Gupta et al., 1992) (i) Direct initiation by FRs produced by

metabolism of the chemical. In this way, CCl_3 derived from CCl can initiate a chain of peroxidation by abstracting a hydrogen from other molecules. (ii) Indirect initiation by the production of reactive forms of oxygen during their metabolism. For example, paraquat can activate O_2 by univalent reduction to the superoxide anion (O_2). (iii) Inhibition of enzymatic systems of defence involved in the control of reactive oxidizing entities. As an example, certain derived compounds of dithiocarbamates behave as inhibitors of Cu-Zn superoxide dismutase (Cu-Zn SOD). (iv) Destruction of natural antioxidants, which control the reactions of peroxidation. Otitoju and Onwurah (2007) reported an elevated lipid peroxidation in rats exposed to permethrin.

Poovala et al., (1999) stated that ROS and concomitant lipid peroxidation, are involved in the toxic effects of bidrin, an organophosphate, on renal cells and that oxidative stress play a role in the structural and functional impairment caused by OP intoxication. Concentration and time-dependent response was studied for lactate dehydrogenase, lipid peroxidation and H_2O_2 content in porcine proximal tubular epithelial cells incubated with bidrin. It was found that all the three parameters were induced in time- and concentration-dependent manner (Poovala et al., 1999).

1.5. Plant cell signaling molecules

Plants produce a wide variety of hormones, which include auxins, gibberellins (GA), abscisic acid (ABA), cytokinins (CK), salicylic acid (SA), ethylene (ET), jasmonates (JA), brassinosteroids (BR) and peptide hormones. Plant hormones play important roles in diverse growth and developmental processes as well as various biotic and abiotic stress responses in plants. Infection of plants with diverse pathogens results in changes in the level of various phytohormones (Adie et al., 2007). Substantial progress has been made in

understanding individual aspects of phytohormone perception, signal transduction, homeostasis or influence on gene expression. Three phytohormones-SA, JA and ET, are known to play major roles in regulating plant defence responses against various pathogens, pests and abiotic stresses such as wounding and exposure to ozone (Balbi and Devoto, 2008).

Salicylic acid (SA) and jasmonic acid (JA) function as signaling molecules in plants, mediating induced plant responses against herbivory and pathogen infection, leading to the activation of genes mediating host plant resistance to insects (Arimura et al., 2005). Both, SA and JA are needed for the plants' induced defense response, and both cause systemic acquired resistance (SAR) to pathogens and mechanical injury in plants (Cohen and Flescher, 2009). SA plays a crucial role in plant defence and is generally involved in the activation of defence responses against biotrophic and hemi-biotrophic pathogens as well as the establishment of systemic acquired resistance (SAR) (Grant and Lamb, 2006). In contrast, JA is associated with defence against necrotrophic pathogens and herbivorous insects. The defence signaling network activated and utilized by the plant is dependent on the nature of the pathogen and its mode of pathogenicity.

Mutants that are affected in the accumulation of SA or are insensitive to SA show enhanced susceptibility to biotrophic and hemi-biotrophic pathogens. Recently, it has been shown that, methyl salicylate, which is induced upon pathogen infection, acts as a mobile inducer of SAR in tobacco (Park et al., 2007). SA levels increase in pathogen challenged tissues of plants and exogenous applications result in the induction of pathogenesis related (PR) genes and enhanced resistance to a broad range of pathogens. SA is involved in regulating plant metabolism and local and endemic disease resistance

in plants in response to pathogen infection (Alverez, 2000). Tobacco mosaic virus (TMV) infection elevates the level of SA in leaves of tobacco plants (Malamy et al., 1990). Similar increase in SA as a result of herbivore attack was shown by GC analysis in tomato plants (Peng et al., 2004). SA induces plant defence responses by altering a mitochondrial enzyme, alternative oxidase, which mediates the oxidation of ubiquinol pool and reduction of oxygen to water, without the synthesis of ATP. This altered activity of alternative oxidase affects the ROS levels in mitochondria, inducing plant defence responses in plants (Norman et al., 2004).

SA and its analogues have shown adverse effects on oxidative phosphorylation and swelling in rat liver mitochondria (Metraux, 2002). It also induces mitochondrial permeability transition (MPT) in the presence of calcium in isolated liver mitochondria (Syeed et al., 2011). Brain and liver mitochondria exhibit morphological alterations such as matrix swelling, decreased matrix density, and loss of cristae in response to SA (Battaglia et al., 2005). Salicylates have damaging effects on isolated mitochondria, causingthe uncoupling of oxidative phosphorylation and swelling (Martens and Lee, 1984; Tonsgard and Getz, 1985). Aspirin and salicylate affect mitochondrial calcium homeostasis and act synergistically with the cation to impair mitochondrial respiration and ATP synthesis (Martens et al., 1986). Again, salicylates inhibit the Krebs cycle enzyme α-ketoglutarate dehydrogenase (Nulton-Persson et al., 2004). Other authors have proposed that the induction of MPT in tumor cells is because of oxidative stress following the production of reactive oxygen species (ROS), elicited by salicylate through a Rac1-NADPH oxidase-dependent pathway (Chung et al., 2003).

JAs are involved in diverse processes such as seed germination, root growth, tuber formation, tendril coiling, fruit ripening, leaf senescence and stomatal opening, they play crucial roles in plant defence responses against insects and microbial pathogens. Several studies have demonstrated that concentrations of JA increase locally in response to pathogen infection or tissue damage and exogenous application of JA induced the expression of defence related genes (Wasternack, 2007). JA causes the induction of proteinase inhibitors in plants (Farmer and Ryan, 1990) and also raises the ROS levels in plants which confer resistance to insect pests. JA is produced quickly following insect feeding (Bi et al., 1997). Tropical application of JA also induces the production of pathogenesis related proteins, and subsequent resistance to phytopathogens (Bi et al., 1997). JA and its methyl ester (methyl jasmonate, MeJa) are key signaling molecules well known for their roles during plant development as well as plant defense and stress responses (Turner et al., 2002; Wasternack 2007; Balbi and Devoto, 2008).

Application of MeJa has also been found to induce the ROS burst in suspension-cultured cells of parsley (*Petroselinum crispum* L.), Taxus (*Taxus chinensis*), Arabidopsis and tobacco Bright Yellow-2 (BY-2) (*Nicotiana tobacum* L.) (Wang and Wu, 2005; Wolucka et al., 2005). Mur et al., (2006) showed that SA and MeJa co-potentiation of the ROS burst is a feature and mechanism of synergistic gene expression and cell death in Arabidopsis and tobacco explants. Based on the accumulated evidences it has been proposed that plant signal molecules are strong candidates for regulation of programmed cell death during pathogen infection (Zhang and Xing, 2008). Recently, the involvement of mitochondria in plant PCD under many stresses has been reported. Yao et al., (2004) showed that the mitochondrial oxidative burst or membrane potential changes are

commonly involved in PCD of Arabidopsis under various stimuli such as ceramide, protoporphyrin IX and the HR elicitor AvrRpt2. In addition, cyt *c* is released from mitochondria in a ROS-dependent manner during heat shock-induced PCD in tobacco BY-2 cells (Vacca et al., 2006), these findings clearly indicate that a mitochondrial function may be shared in a very similar way during PCD in both animals and plants. Therefore, it is tempting to speculate that mitochondria might be the epicenter of JA-induced cell death in plants and animals.

Recent studies have shown that jasmonates can suppress proliferation of human cancer cells, and one of the probable reasons may be mitochondrial dysfunction, consisting of mitochondrial membrane permeability transition, dissipation of inner membrane potential, osmotic swelling of matrix and release of cyt *c* (Biban et al., 1995). Jasmonates increased significantly the survival of lymphoma-bearing mice and induced death in human leukemia, prostate, breast and melanoma cell lines (Fingrut and Flescher, 2002), as well as in leukemic cells from chronic lymphocytic leukemia patients (Rotem et al., 2005). The mechanism of action of jasmonates involves direct action on mitochondria, resulting in cell death (Rotem et al., 2005). MeJa-induced ROS production has also been implicated as one of the mechanisms by which MeJa induces cancer cell death. For example, MeJA induces apoptosis in A549 human lung adenocarcinoma cells through induction of the expression of pro-apoptotic members of the Bcl-2, Bax and Bcl-XS protein families and the activation of caspase-3 via ROS production (Kim et al., 2004; Oh et al., 2005).

In animals, the mitochondrion integrates diverse cellular signals and initiates the death execution pathway. The application of pro-death stimuli to mammalian cells leads

to loss of mitochondrial transmembrane potential (MTP), changes in mitochondrial morphology and the release of cyt *c*, which initiates several downstream processes (such as the activation of cell-degrading caspase proteases) that culminate in cell death (Liu et al., 1996; Zou et al., 1997; Wang, 2001). It has been proposed that MeJa provokes death in cancer cells, not by changes in cellular mRNA transcription, protein translation or p53 expression, but by acting directly and selectively on mitochondria (Rotem et al., 2005). Also in this study, evidence provided has demonstrated that MeJa could induce mitochondrial swelling and mitochondrial membrane depolarization via abnormal opening of the mitochondrial permeability transition pore (MPTP) complex channel and therefore provoke cyt *c* release from mitochondria to the cytosol and ATP depletion, leading ultimately to cancer cell death (Rotem et al., 2005).

1.6. Dihydrolipoamide dehydrogenase

Glucose is hydrolysed to yield energy in all organisms through glycolysis finally yielding pyruvate. Pyruvate is further metabolized by oxidative decarboxylation by the pyruvate dehydrogenase complex (PDHC) (Hein and Steinbuchel**,** 1994) to yield the central intermediate acetyl-CoA, which is then oxidized in the Krebs cycle or used in anabolic pathways, like synthesis of essential cell constituents. The PDHC belongs to the family of 2-oxoacid dehydrogenase multienzyme complexes, which irreversibly convert 2-oxoacids to the corresponding acyl-CoA derivates with concomitant formation of CO_2 and reduction of NAD^+ to NADH. Currently, three different complexes of this family are known: (i) The PDHC, (ii) the 2-oxglutarate dehydrogenase complex (OGDHC), and (iii) the branched-chain dehydrogenase complex (BCDHC) for degradation of branched-chained amino acids (Berg and de Kok, 1997). In addition, the glycine dehydrogenase

complex (GDHC) (Douce et al., 2001) and the acetoin dehydrogenase complex (ADHC) (Oppermann et al., 1991) have related structures or catalyze related reactions, respectively. Except for the GDHC, these multienzyme complexes share architectural features and consist of three principal enzyme components: a substrate-specific decarboxylase/dehydrogenase (E1), a complex-specific dihydrolipoamide acetyltransferase (E2), and a nonspecific dihydrolipoamide dehydrogenase (E3) (Aevarsson et al., 1999; Knapp et al., 2000). Aggregation of these three components results in the formation of a core complex. While the subunit conformations of E1 and E2 admittedly differ (Schreiner et al., 2005), the nonspecific E3 component is always arranged as a homodimer (Pratt et al., 2005). E3 is also a component of the glycine cleavage system (Kikuchi and Higara, 1982), another mitochondrial enzyme complex, where it is known as protein L.

In the PDHC, pyruvate is first decarboxylated by the pyruvate decarboxylase (E1) with formation of the intermediate hydroxyethyl thiopyrophosphate. Its hydroxyethyl moiety is then transferred to lipoamide, with its concomitant oxidation yielding acetyl dihydrolipoamide. This second step is also mediated by E1. The acetyl moiety is transferred in a third step to CoA by the dihydrolipoyl transacetylase (E2) component, and acetyl-CoA is released. The reoxidation of dihydrolipoamide formed in the second step is secured in the fourth and fifth steps by the dihydrolipoamide dehydrogenase (E3), which reduces NAD^+ via flavin adenine dinucleotide (FAD) (Patel and Korotchkina**,** 2003).

Dihydrolipoamide dehydrogenases (DHLDHs) belong to the class of flavoenzymes and the family of pyridine nucleotide-disulfide oxidoreductases and show a typical domain structure. Each subunit of a DHLDH consists of four domains: the FAD-binding domain, the NAD-binding domain, the central domain, and the interface domain. In the homodimer, the domains of both subunits partially interact with each other; therefore, dimerization is essential for an active enzyme (Mattevi et al., 1992; Rajashankar et al., 2005). Subunits E_1 and E_2 are substrate specific and differ in each complex, whereas E_3 is identical in all the three complexes, catalyzing the reversible reaction: Lipoamide + NADH+H^+ ↔ Dihydrolipoamide + NAD^+ (Lohrer and Krauth-Siegel, 1990). All DHLDHs are stable homodimers, each monomer possessing a noncovalently but tightly bound flavin adenine dinucleotide (FAD), a transiently bound NADH or NAD^+ molecule, and two redox active cysteine residues (Brautigam et al., 2005). Apart from its role in α-ketoacid dehydrogenase complexes, E_3 exhibits a number of additional functional capacities. DHLDH acts as a diaphorase (Patel et al., 1995), it is also capable of scavenging nitric oxide (Igamberdiev et al., 2004) and can serve as an antioxidant by protecting other proteins against oxidative inactivation (Korotchkina et al., 2001). DHLDH can also act as a proteolytic enzyme when the stability of the enzyme is altered (Babady et al., 2007).

The enzyme has been purified from a number of prokaryotes, eukaryotes, and some archaebacteria. Lipoamide dehydrogenase isolated from the crude extracts of *Streptomyces seoulensis* (Youn et al., 1998), composed of two identical subunits with a molecular mass of 54 kDa and contained 1 mol of FAD per mol of subunit, with absorption maxima of flavoprotein at 272, 349, and 457 nm. The reaction of the enzyme

catalyzed the reaction by a pingpong mechanism. The structural gene for the enzyme was cloned using a DNA fragment using PCR. Dietrichs and Andreesen (1990) purified DHLDH from the anaerobic glycine-utilizing bacteria *Clostridium cylindrosporum*, *Clostridium sporogenes*, and *Peptostreptococcus glycinophilus*, which were almost similar in their properties with differences in their specificity towards coenzyme. DHLDH isolated from cyanobacteria, *Anabaena* sp. strain P.C.C. 7119, was also a dimer, FAD-containing protein with a native molecular mass of 104 kDa, and suggested that the LPD in *Anabaena* might be involved in a glycine cleavage system (Serrano, 1992). DHLDH of human (Babady et al., 2007), *Mycobacterium tuberculosis* (Rajshankar et al., 2005), silk worm (Huo et al., 2010), and *Manduca sexta* (Pullikuth and Gill, 1997) were expressed and purified from *Escherichia coli* for the structural and functional studies.

DLDHs of soybean and *Haloferax volcanii* were also expressed and purified from *E. coli* for characterization of the enzyme (Connaris et al., 1999; Moran et al., 2002). Coupled mitochondria maintain a pH gradient such that its internal, or matrix pH is more alkaline than cytosol and that the substrate specificity and oligomeric state of DHLDH isolated from Pig heart varies with pH (Klyachko et al., 2005). Conditions known to destabilize the DHLDH homodimer enabled the mouse, pig, and human enzyme to function as a protease (Barbady et al., 2007). DHLDH expression was studied in post natal development and aging in rat (Yan et al., 2008).

Apart from its role in α-ketoacid dehydrogenase complexes, E_3 exhibits a number of additional functional capacities. DHLDH isolated from porcine heart catalyzes NADH-dependent scavenging of nitric oxide (Igamberdiev et al., 2004). The reaction consumes NADH and has been reported to possess a K_m of 0.5 µM for nitric oxide.

DHLDH can serve as an antioxidant by protecting other proteins against oxidative inactivation (Korotchkina et al., 2001). DHLDH can also act as a proteolytic enzyme when the stability of the enzyme is altered (Babady et al., 2007). The enzyme is known for its broad specificity for different electron acceptors (Savage, 1957; Massey, 1960), the importance of these reactions has been discussed in many contexts ranging from detoxification of injurious chemical species to converting normally protective and vital functions of the enzyme into cytotoxic ones. In addition to the activity with lipoamde, it can act as a diaphorase, transferring electrons from NAD(P)H to oxygen. It is also capable of using artificial electron acceptors such as methylene blue, DCPIP, ferricyanide or quinines (Massey, 1960; Ide et al., 1967). It is also known for at least some DHLDHs, that they can use cyt *c* as an electron acceptor (Youn and Kang, 2000). DHLDH also catalyzes transhydrogenase reactions with pyridine nucleotides (Ide et al., 1967).

Because of the critical nature of this enzyme in linking glycolysis to both the TCA cycle and fatty acid biosynthesis, the enzyme is selectively inhibited in trypanosoma (Friedheim, 1994). Lipoamide-reducing activity was reversibly inhibited by sodium arsenite, with no decrease in the diaphorase activity in *Mycobacterium smegmatis* (Marcinkeviciene and Blanchard, 1996).

1.7. Insecticide resistance in *H. armigera*

Insecticides had been found very effective for the control of chewing and sucking insect pests in the early 1980s. The development of resistance to pesticides in arthropods is one of the main drawbacks of the chemical control of agricultural pests and vector species that give rise to public health concerns (Denholm et al., 1998; Hemingway and Ranson, 2000). This phenomenon has critical implications in pest management, as

chemical measures remain central to pest control plans, especially when insect density, health risks and the consequent economic damage are all high (Casida and Gary, 1998). Indiscriminate applications of pesticides resulting from reduced levels of control in resistant populations may lead to a higher environmental load. Therefore, the evolution of pesticide resistance must be slowed by making optimal use of existing compounds and improving our knowledge about the interaction between cells and toxic substances in order to find novel target sites for compounds with low, if any, environmental impacts (Kogan, 1998). However, with their extensive use, a widespread resistance to insecticides occurred in *H. armigera* in India in 1990s. Existence of resistance to pyrethroids, organophosphates, carbamates and cyclodienes were also reported (Dhingra et al., 1998; Armes et al., 1994; Kranthi et al., 2001). *H. armigera* shows resistance to almost all pesticides which are applied to it, for example, resistance to organophosphates, carbamates and pyrethroids (Mahmood, 2007). The insecticide resistance levels in *H. armigera* from 2001-2005 from the main cotton-growing regions of Central and South India are presented in Table 1.2 (Chaturvedi, 2007).

Table 1.2: Response of field strain *H. armigera* for different insecticides in India (Chaturvedi, 2007).

Insecticides	**Resistance fold**
Fenvalarate	11-245
Cypermethrin	49-712
Endosulfan	13-79
Monocrotophos	2-50
Chlorpyrifos	1-38
Methomyl	1-49
Quinolfos	13-182

RF: LD_{50} of resistant strain / LD_{50} of susceptible strain.

Several factors responsible for insecticide resistance have been identified in the insect pest, *H. armigera*, including insecticide insensitivity of acetylcholinesterase, Esterase and phosphatase-mediated organophosphorous resistance and P-glycoprotein (Srinivas et al., 2004).

1.7.1. Multidrug resistance

Multidrug resistance is a condition enabling a disease-causing organism to resist distinct drugs or chemicals of a wide variety of structure and function targeted at eradicating the organism. Organisms that display multidrug resistance can be pathologic cells, including bacterial and neoplastic (tumor) cells. When an organism is resistant to more than one drug, it is said to be multidrug resistant. Multidrug trasport systems can be devided into two major classes. ATP- binding cassette (ABC) transporters utilize the release of phosphate bond energy by ATP hydrolysis, to pump drugs out of the cell (Higgins, 1992). Secondary transporters mediated the extrusion of drugs from the cell in a coupled exchange with ions, in the absence of a chemical reaction (Paulsen et al., 1996).

1.7.2. ATP-binding cassette transporters

ATP binding cassette (ABC)-transporter are members of a protein superfamily that is one of the largest and most ancient families with representatives in all extant phyla from prokaryotes to humans (Jones and George, 2004). ABC transporters are transmembrane proteins that utilize the energy of adenosine triphosphate (ATP) hydrolysis to carry out certain biological processes including translocation of various substrates across membranes and non-transport-related processes such as translation of RNA and DNA repair (Davidson et al., 2008). They transport a wide variety of substrates

across extra- and intracellular membranes, including metabolic products, lipids and sterols, and drugs. Proteins are classified as ABC transporters based on the sequence and organization of their ATP-binding cassette (ABC) domain(s). ABC transporters are involved in tumour resistance, cystic fibrosis, bacterial multidrug resistance, and a range of other inherited human diseases.

1.7.3. P-glycoprotein

Overexpression of permeability or phosphorylated or plasma membrane glycoprotein, known as P-glycoprotein (abbreviated as P-gp or Pgp) is a ATP dependent drug transporter, is a well-characterized ABC-transporter of the MDR/TAP subfamily (Michael, 2001). P-gp is also called ABCB1, ATP-binding cassette sub-family B member 1, MDR1, and PGY1. P-glycoprotein has also recently been designated CD243 (cluster of differentiation 243). In humans, P-glycoprotein is encoded by the *ABCB1* gene (Ueda et al., 1987) Pgp is extensively distributed and expressed in the intestinal epithelium, hepatocytes, renal proximal tubular cells, adrenal gland and capillary endothelial cells comprising the blood-brain and blood-testis barrier.

In the mid-1980, P-glycoprotein (Pgp) was revealed to be the molecular culprit in a pernicious problem encountered in cancer chemotherapy: multiple drug resistance (MDR). MDR is evident when a tumor being treated with an anticancer drug becomes resistant not only to the drug used in the therapy, but to a wide range of other drugs that are not similar in structure or function. MDR thus severely curtails the therapeutic options for a patient. The list of chemotherapeutic drugs rendered ineffective by MDR includes vinblastine, daunomycin, and actinomycin D (Juranka et al., 1989). Not only are these drugs structurally dissimilar, but they have different intracellular targets. Traits that

the drugs have in common are that they are large, hydrophobic, aromatic, nitrogenous bases, and they are isolated from natural products. Because of the obvious clinical relevance of the phenomenon, MDR and Pgp have been intensively studied in the last 10 to 15 years. Laboratory studies of the MDR phenomenon were facilitated by the development of cell lines displaying the MDR phenotype (Gottesman and Pastan, 1988). Kinetic experiments with these cells indicated that an energy-dependent efflux mechanism maintains intracellular concentrations of the drugs at non-toxic levels. Molecular analysis indicated that these cells were consistently over expressing a 170-kDa glycosylated protein in the plasma membrane (Endicott and Ling, 1989). This protein was dubbed P-glycoprotein (P for its perceived ability to alter the permeability of drugs). The expression of this integral protein correlated positively with a degree of drug resistance, and negatively with intracellular drug accumulation (Endicott and Ling, 1989). Transfection and expression of the Pgp cDN.4 were shown to be sufficient to cause drug sensitive cells to become drug resistant (Gros et al., 1986). Genetic analysis of human (Roninson et al., 1986) and hamster (Roninson et al., 1984) resistant cell lines showed that MDR was associated with amplification of one or more genes. The amplified fragments were shown to contain genes that coded for P-glycoprotein (Riordan et al., 1985; Chen et al., 1986).

1.7.4. Mechanism of action of P-glycoprotein

Pgp is a member of a family of membrane-spanning proteins, most of which are transporters and all of which bind ATP to sites conserved throughout the family. Consequently, they are known as ATP Binding Cassette (ABC) proteins, with eight subfamilies, designated ABCA to ABCH. Pgps are within the ABCB subfamily

(Allikmets et al., 1996). Their structure is of two homologues, but non-identical halves, each of six transmembrane domains and an intra-cytoplasmic loop encoding an ATP-binding site (Gottesman and Pastan, 1998). Electron microscopy and single particle image analysis have shown the structure of human Pgp to consist of a cylinder with a diameter of about 10 nm and height of 8 nm, appearing toroidal (doughnut-shaped) when viewed from the extracellular surface. A large pore (5 nm) is open at the extracellular end, but closed at the intracellular face, forming a conical chamber. A second asymmetrical pore opens within the membrane bilayer (Rosenberg et al., 1997).

The precise mechanism of Pgp action is still unclear. However, the ‘‘flippase’’ model of (Higgins and Gottesman, 1992) appears to be supported by the evidence so far. In this model, a substrate, either in the cytoplasm or within the lipid bilayer, binds to a chamber within the Pgp molecule. Conformational changes then ‘‘flip’’ the substrate to the extracellular medium. Energy for this process would be provided by the hydrolysis of ATP, a known requirement of Pgp action. Whether the two halves of the molecule operate in turn (Senior et al., 1995) or in cooperation (Locher et al., 2002) is disputed.

A distinctive character of Pgp is the huge range of substrates transported, with one recent survey listing 84 compounds alone (Oesterheld, 2003). Examples included anti cancer drugs, alkaloids, steroid hormones, HIV protease inhibitors and antibiotics. However, these substrates tend to share a number of chemical characteristics. Substrates tend to be moderately lipophilic (Hofsli and Nissen-Meyer, 1990), with a molecular mass over 300 Da (Biedler and Riehm, 1970). A comparison of the physical properties of 44 compounds in relation to their transport by Pgp found good predictive properties for transport to be (i) at least one cyclic structure of 6 sides or more, (ii) a molecular mass of

over 400 Da, (iii) moderate lipophilicity (Bain et al., 1997). A further study of 100 compounds concentrated on the presence and spatial separation of electron donor groups on the substrate surface, suggesting that transport is proportional to the number and strength of hydrogen bonds formed between the substrate and transmembrane domains of the Pgp molecule (Seelig, 1998).

An interesting property of Pgp is that substrate transport can be inhibited through the action of secondary compounds, allowing accumulation of the primary compound in the cell. As cells are effectively sensitized to toxins, they were formerly able to resist, this phenomenon is known as “chemosensitisation”. Because of clinical interest in inhibiting MDR in tumors, many chemosensitizers have been identified (over 100 at a time of writing). Although varying considerably in structure they are all lipophilic, and most are heterocyclic and positively charged (Ford, 1996).

Although P-gp is generally considered to be localized in the cytoplasmic membrane and to mediate drug resistance by lowering total intracellular drug concentrations (Stride et al., 1999), some reports have suggested a more complex subcellular distribution of this protein as well as for other drug transporters that belong to the ATP-binding cassette family of proteins (Marquardt et al., 1990; Shapiro et al., 1998; Meschini et al., 2000). P-gp is also expressed in the membrane of the nucleus of MDR cell lines selected by doxorubicin, suggesting its possible involvement in the extrusion of the drug from the nucleus of resistant cells (Maraldi et al., 1999). Moreover, P-gp was described in the intracellular membranes (Gong et al., 2000). In particular, significant levels of P-gp were found in intracytoplasmic vesicles, identified as part of the Golgi apparatus, in different resistant cell lines, suggesting that P-gp exerts its function also in

this region (Meschini et al., 2000; Gervasoni et al., 1991; Rutherford and Willingham, 1993). Development of a P-gp-mediated MDR phenotype can be associated with several changes in cell biology of resistant cells (Breier et al., 2005).

1.8. Storage proteins

The fat body is a relatively large organ distributed throughout the insect body, preferentially underneath the integument and surrounding the gut and reproductive organ (Dean et al., 1985).The insect fat body plays an essential role in energy storage and utilization. It is the central storage depot for excess nutrients. In addition, it is an organ of great biosynthetic and metabolic activity (Law and Wells, 1989). Fat body cells not only control the synthesis and utilization of energy reserves-fat and glycogen-but also synthesize most of the hemolymph proteins and circulating metabolites. Large amounts of relevant proteins, such as storage proteins used as an amino acid reservoir for morphogenesis, lipophorins responsible for the lipid transport in circulation, or vitellogenins for egg maturation, are secreted by the fat body (Keeley, 1985). Most of the insect's intermediary metabolism takes place in this organ, including lipid and carbohydrate metabolism, protein synthesis, and amino acid and nitrogen metabolism. Some metabolic processes are stage specific such as the synthesis and secretion of storage proteins into the hemolymph that occur in the feeding larva or the synthesis of vitellogenin in adult insects.

Storage proteins are the major nutrient components important for growth and development; they are synthesized by fat body, secreted into the larval hemolymph and then reabsorbed by fat body shortly before pupation (Dong et al., 1996; Kim et al., 2004). These proteins are initially stored as protein granules within the pupal fat body and are then proteolytically degraded to serve as sources of amino acids (Tungjitwitayakul et al., 2008).

A wide variety of insects synthesize special storage proteins during periods of resource abundance. These are retained until the time and setting required for egg production or other functions including cuticle formation, transport of organic compounds, and humoral immune defense (Burmester, 1999). For example, queen ants of several species have large amounts of large hexameric (e.g., comprised of six subunits) proteins that are digested to produce food for their first larvae during claustral colony founding (Wheeler and Martinez, 1994, 1995; Wheeler and Buck, 1995). Adult worker honey bees have been documented as containing Hex7Oa in relatively large amounts (Danty et al., 1998). In addition to hexamerins, there are additional proteins that may serve as protein storage molecules, including vitellogenin which is very high density lipoproteins (VHDLs) (Wheeler and Buck, 1995; Amdan and Omholt, 2002). Storage proteins play diverse roles in adult social Hymenoptera, which are often characterized by long lifetimes as well as by group living and division of reproductive effort. Koehler (1921) reported that protein is present in large quantities in the fat body of worker honeybees early in the temperate zone winter but that by spring most of that protein is gone. Martinez and Wheeler (199l, 1993) showed that storage proteins accumulate in hemolymph and fat body of worker ants held in queenless groups. In new queens of claustrally founding ants, which do not forage for food, storage proteins are present (Martinez and Wheeler, 1994; Wheeler and Buck, 1995) and play a significant role in provisioning the queen's first brood (Martinez and Wheeler, 1994; Wheeler and Buck, 1996).

The first storage proteins to be described in lepidopterans were two methionine-rich hexamerins from *Hyalophora cecropia* (Tojo et al., 1978). They differed in electrophoretic mobility in native PAGE and also in amino acid composition, methionine contents being 7.0% for the more slowly migrating and 4.9% for the faster. The two

appear in the hemolymph late in the last larval instar, rise to maximum concentrations early in the larval-pupal molt, and are then largely endocytosed by the cells of the fat body, which store them in crystalline form until they are utilized during adult development. Methionine-rich hexamerins have been identified in a variety of moths, either as isolated proteins or from cDNA sequencing (Tojo et al., 1978, 1980, 1985).

A third lepidopteran hexamerin, first described in *Manduca sexta* (Kramer et al., 1980), has a high aromatic amino acid content, resembling in this regard one of the prototypical hexamerins of Calliphora. The importance of aromatic amino acids in sclerotization led to the suggestion that hexamerins in this class, now known as arylphorins (ArH) (Telfer et al., 1983), are adapted to support cuticle deposition. While many lepidopterans produce just these three, some also contain a riboflavin-binding hexamerin, RbH (Miller and Silhacek, 1992; Magee et al., 1994). Like ArH, RbH is a major component of pupal hemolymph that disappears during adult development (Pan and Telfer, 1999).

The overall characteristics in lepidopteran aryphorins resemble those of the dipteran proteins, but several important differences exist. Lepidopteran arylphorins have a similar percentage of aromatic amino acids, but are relatively low in methionine. The sequence identities between those lepidopteran arylphorins sequenced (*Manduca sexta*, Willot et al., 1989; *G. mellonella*, Memmel et al., 1992; *Bombyx mori*, Fujii et al., l989) is between 50 and 70%, but they are much less homologous to the dipteran arylphorin. While arylphorin is synthesized at high rates in fat body of last instar larvae and released into the hemolymph, evidence exists that the protein is expressed at a low rate throughout larval life (Ray et al., 1987; Webb and Riddiford, 1988), and also in the gonads of adult

insects (Miller et al., 1990; Kumaran et al., 1993). Nevertheless, high hemolymph concentrations are found only during the last larval instar and re-absorption into fat body begins in prepupa. In most cases, arylphorin is not completely removed from the hemolymph, possibly due to its high concentration (Haunerland et al., 1990). In detailed studies the uptake and accumulation into fat body has been biochemically and electron microscopically documented in *Hyalophora cecropia* (Tojo et al., 1978), *B. mori* (Tojo et al., 1980) and *Helicoverpa zea* (Wang and Haunerland, 1991 and 1992). Arylphorin has been positively identified in crystalline protein granules in fat body and it appears that these granules are gradually, but not completely broken down during the pupal stage; in fact, many granules have been detected in adult fat body and it has been suggested that arylphorin may also serve as an amino acid source for yolk protein production (Wang and Haunerland, 1991).

As for the place of synthesis and storage, most studies have not attempted to distinguish between different regions of fat body. Detailed studies have only been perforded in *H. Zea*, where, aided by a colored storage protein, a clear distinction could be made between the place of synthesis and storage (Haunerlund and Shirk, 1995). Arylphorin is synthesized in the larval fat body that is found peripherally, between the outer muscle layer and the cuticle of 5th instar larvae. This white tissue, however, does not sequester arylphorin, instead the protein is actively taken up by newly formed fat body tissue that is located centrally, within the body cavity, surrounding the gut, from electron micrographic studies it is now clear that this perivisceral fat body persists through the pupal stage and develops into the adult fat body; the peripheral fat body, however, decays. Ryan et al., (1985) cited a number of studies suggesting diverse roles

for the arylphorins, such as serving as a reservoir of tyrosine for cuticle sclerotization, a protein component of the cuticle, and a carrier for ecdysteroids and xenobiotics. Kramer et al., (1980) first isolated and characterized *M. sexta* arylphorin (originally termed Manducin) from hemolymph of 5th instar larvae. In this study, it was determined that *M. sexta* arylphorin was a hexameric protein rich in aromatic amino acids with a molecular weight of approximately 450,000, and consisting of two distinct subunits. Webb and Riddiford (1988) found that *M. sexta* arylphorin is expressed primarily by the fat body of late instar larvae, with lesser levels of expression occurring in salivary gland, muscle, and epidermis.

1.9. AIMS AND OBJECTIVES

Lepidopteran larvae in general are 'eating machines', and grow much faster than the young mammals and birds with highly active metabolic processes (Chamberlin, 2004). This phenomenal growth occurs due to the large midgut epithelium, which efficiently helps in digestion and absorption of the nutrients from the consumed food. Because of its reliance on aerobic metabolism, it also requires a highly active mitochondrial system for oxidative phosphorylation to meet the energy demands of the insect undergoing metamorphosis. This metabolic system conceptually divides oxidative phosphorylation into three reaction blocks: the substrate oxidation system, which includes metabolite transporters, the citric acid cycle, and the ETC; 2) the phosphorylation system, which includes, F_0F_1 ATP synthase, the phosphate transporter, and the adenine nucleotide translocase; and 3) the proton leak, which includes the passive cation cycles across the inner mitochondrial membrane (Chamberlin, 2006). Oxidative phosphorylation in insects system is studied relating to metamorphosis in tobacco hornworm by Chamberlin (2004, 2006).

In addition to the their neurotoxicity, insecticides have been reported to cause a number of other biochemical afflictions such as, altered energy metabolism, oxidative stress, mitochondrial respiratory chain dysfunction and DNA damage in different non-target organism including human (Gupta et al., 2001; Rai and Sharma, 2007). Organophosphorous insecticides (OPI) induced oxidative stress, genotoxicity and DNA damage in human (Shadnia et al., 2005), chromosomal aberrations, alterations in oxidative phosphorylation, and carbohydrate metabolism in fish (Das and Jon, 1999). Several insecticides have been shown to affect mitochondrial bioenergetics.

Methylparathion, carbofuran, permethrin, and fenvalarate are extensively used to control *H. armigera* (Sharma, 2005). The present studies were undertaken to evaluate the effect of these insecticides in inducing mitochondrial dysfunction and oxidative stress, which could affect the growth and development of *H. armigera*.

Plants are continuously exposed to the challenge of a variety of herbivores. However, to defend themselves against the herbivores, plants have evolved constitutive and inducible defense mechanisms. Salicylic acid (SA), Jasmonic acid (JA), nitric oxide (NO) and ROS (particularly H_2O_2) increase in abundance following pathogen recognition and each are important signaling molecules that promote and coordinate defense and HR responses (Goggin, 2007; Sasan et al., 2007). Since the level of these signaling molecules increases during insect and pathogen attack, as well as exogenous application, induces defense mechanism in plants. The present studies were therefore undertaken to determine whether SA and JA could have a direct effect on mitochondrial oxidative phosphorylation and the respiratory chain complexes of *H. armigera.*

The mitochondrial enzyme, dihydrolipoamide dehydrogenase (E.C.1.8.1.4) belongs to the group of flavin containing pyridine nucleotide disulfide oxidoreductases (Huo et al., 2010) is a component of many multienzyme complexes. Although DHLDH has been purified from many prokaryotes and eukaryotes, it has been characterized only from limited number of insect species. Since insects represent the most abundant and diverse group of organisms in any Phylum, their number and diversity far exceeds the species in all other Phyla combined (Novotny et al., 2002), they are interesting candidates to study evolutionary, genetic and biochemical properties of multi-enzyme complexes. Because of the amenability of insects to adapt environment and develop resistance to

several insecticides, biochemical properties of the enzymes involved in metabolism, such as DHLDH, could be helpful to develop insecticidal molecules for the control of *H. armigera*, with a different mode of action.

Insecticide resistance in *H. armigera* is due to the combined effects of insensitivity of acetylcholine esterase to insecticides, expression of higher levels of esterases, phosphatases and a specific protein called p-glycoprotein ATPase (Srinivas et al., 2004). Insects exhibiting resistance to one group of insecticides generally develop resistance to other classes of insecticides, a phenomenon often referred to as cross-resistance. The role of Pgp-like proteins in insects merits a study because such transporters may contribute to insecticide resistance. In view of the toxicity of the insecticides to the mitochondria and development of resistance to insecticides in *H. armigera*, the present studies were undertaken to gain an understanding of the role of mitochondrial Pgp ATPase in the efflux of xenobiotic compounds and providing protection to the cell organelle in insecticide-resistant *H. armigera* larvae from the insecticides.

A characteristic family of proteins has been found in storage tissues such as the hemolymph and fat body of all insect species. These storage proteins have been found to play a major role in insect metamorphosis to pass through different instars and reproduction. Now a days disruption of the regulation of storage proteins is a potential method to control this pest.

The present studies were aimed to investigate the following objectives:

- To evaluate the effect of insecticides on mitochondria and oxidative stress in *H. armigera.*
- To evaluate the interaction of plant cell signaling molecules, SA and JA, with the mitochondria and their effects on oxidative stress in *H. armigera.*
- Characterization of dihydrolipoamide dehydrogenase, a metabolic enzyme from mitochondria of *H. armigera.*
- Detection of P-gp from the mitochondria of insecticide resistant strains of *H. armigera.*
- Characterization of arylphorin, a storage protein from the hemolymph of *H. armigera.*

Chapter II

Insecticides-induced mitochondrial dysfunction and oxidative stress in Helicoverpa armigera

2.1. INTRODUCTION

Insecticides exhibit a high level of pest control ability combined with a relatively low degree of environmental toxicity; hence, they are used widely around the world in agriculture and in households. Almost all classes of insecticides are neurotoxic in nature with their toxic effects employing on broad group from insects to mammals. There are a number of reports on the mode of action of insecticides on insects as well as on non-target groups with different target sites. Studies on the effects of insecticides on insect pests have mainly been carried out under *in vivo* conditions in rodents (Piña-Guzmán et al., 2005), Pisces (Li and Zhang, 2002), and pigs (Campagna et al., 2002). However, there is little information on the effect of insecticides in insects under *in vivo* conditions, and hence, we evaluated the *in vivo* effects of methylparathion, carbofuran, permethrin and fenvalarate on the larvae of cotton bollworm, *H. armigera*.

Several insecticides have been shown to affect mitochondrial bioenergetics. Pesticides adversely affect energy mitochondrial metabolism in fish (Reddy and Phillip, 1992) and mammals (Gassner et al., 1997). Effect of deltamethrin, a pyrethroid, was studied on rat liver mitochondria, and it is proposed that it inhibited oxidative phosphorylation, caused alterations in membrane permeability, and induced mitochondrial swelling (Braguini et al., 2004). Induction of oxidative stress is also one of the main mechanisms of action of many insecticides. Exposure to insecticides induces superoxide, H_2O_2 and alters the levels of antioxidant enzymes in mice (Olgun and Misra, 2006). As mitochondria are the major sources of reactive oxygen species in cells, the present studies were undertaken to understand the effect of methylparathion, carbofuran, permethrin and fenvalarate in inducing mitochondrial dysfunction and oxidative stress,

which could affect the growth and development of *H. armigera*. This information will be useful to develop strategies for managing development of resistance to insecticides in *H. armigera* for sustainable crop protection.

2.2. MATERIAL AND METHODS

2.2.1. Glassware cleaning

Glassware was first cleaned in tap water and soaked in chromic acid solution (15 % potassium dichromate in 25 % concentrated sulfuric acid) for 12 h. This was followed by thorough tap water washing in detergent solution. The glassware was then dried in hot air oven after successive washings with deionized and distilled water.

2.2.2. Chemicals

NADH, bovine serum albumin, and ADP were purchased from Sigma Aldrich (Mumbai, India). Sucrose was purchased from Qualigens (Mumbai, India). Methylparathion (99.3%) and carbofuran (99%) were procured from Pesticide Analysis Laboratory, Gulbarga, India. Permethrin (99%) and fenvalarate (99.4%) from Dow Agrosciences were gifted from Dr Sharom, Department of Environmental Biology, University of Guelph (Guelph, ON, Canada). The other chemicals used in these studies were of analytical grade.

2.2.3. Insect culture

Larvae of *H. armigera* were obtained from the insect rearing laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. The larvae were reared on a chickpea based semi-synthetic diet under laboratory conditions at 27 ± 1 ˚C, 65 ± 5% RH, and 12 h photoperiod (Armes et al., 1992). The *H. armigera* neonates were reared in groups of 200 to 250 in 200 ml

plastic cups (having 2 to 3 mm layer of artificial diet on the bottom and sides) for five days. After days, the larvae were transferred individually to six cell well plates (each well 3.5 cm in diameter, 2 cm in depth) to avoid cannibalism. Each cell well had sufficient amount of diet (7 ml) to support larval development until pupation. The pupae were removed from cell wells, sterilized with 2% sodium dithionite solution, and kept in groups of 50 in plastic jars containing vermiculite. Upon emergence, ten pairs of adults were released inside an oviposition cage (30 x 30 x 30 cm), and were provided with 10% sucrose or honey solution on a cotton swab as food. Diaper linders, which have a rough surface, were hung inside the cage as an oviposition substrate. The liners were removed daily and the eggs sterilized in 2% sodium hypochlorite solution. The liners were dried under fan and then placed inside the plastic cups with artificial diet. After eggs hatching, the larvae moved to artificial diet, and the liners were removed after 4 days. Neonate larvae were used for bioassays using diet impregnation assay experiments (Sharma, 2005).

Artificial diet for rearing *H. armigera* and for bioassay was prepared as follows:

1. Measured quantities of chickpea flour (75 g), ascorbic acid (1.175 g), sorbic acid (0.75 g), methyl-4-hydroxy benzoate (1.25 g), aureomycin (2.875 g) and yeast (12 g) were taken in a bowl and 112.5 ml of warm water was added, and mixed thoroughly using a blender.

2. Then 1 ml of formaldehyde and 2.5 ml of vitamin stock solution [Nicotinic acid (1.528 g), riboflavin (0.764 g), aneurine hydrochloride (0.382 g), pyridoxine hydrochloride (0.382 g), folic acid (0.382 g), D-Bioterin (0.305 g), and cyano cobalamine (0.003 g) were mixed in 500 ml of water] were added and were mixed well.

3. In a separate container 4.325 g of agar-agar was added to 200 ml of water container and boiled for 5 min.

4. The agar-agar solution was added to the other diet ingredients, and was mixed thoroughly in a blender to get an even consistency.

5. The diet so prepared was poured into small plastic cups and allowed for cooling under a laminar flow chamber for 1 to 2 h.

6. Around 300 ml of diet was found sufficient to rear 30-neonate larvae up to pupation.

2.2.4. Isolation of mitochondria

The fourth- and fifth-instar larvae were starved for 3 h, their midguts content removed, washed in cold distilled water, and then homogenized in dounce homogenizer under cold conditions in 0.25 M sucrose solution containing 0.1% defatted bovine serum albumin (BSA). The homogenate was filtered through a moist muslin cloth, the filtrate centrifuged at 800 xg for 10 min at 4˚C. The residue was re-suspended in extraction buffer, and centrifuged at 800 xg for 5 min. The supernatants from both the centrifugations were combined and centrifuged at 8,000 xg for 10 min. The mitochondrial pellet was re-suspended in the reaction mixture, and used immediately for measuring oxygen consumption (Chamberlin, 2006).

2.2.5. Protein estimation

Protein concentration was determined by Lowry's method (1951) using BSA as a standard.

2.2.6. Oxygen electrode unit

The Hansatech D.W. units (Hansatech Instruments Limited, Bachofer, Reutlingen 72734, Germany) contain a Clark-type O_2 electrode, it is supplied with electrode disc, which is located in the floor of a water-jacketed reaction chamber, a magnetic flea, and a

CB1-D3 control box. The control box is connected to 12 V dc supply. A fine paper spacer such as 1" square piece of tissue, preferably cigarette paper is placed over the platinum cathode disc and moistened with 50% saturated KCl. The same solution is also used to flood the well which houses the silver anode. A 1" square of PTFE membrane on the top of the spacer and an O ring applied. The control box is connected with the reaction chamber and external battery and the instrument is switched on and allowed to stabilize till the reading steadily reduced to a stable reading in the region of 400-800 mV. The electrode signal is set back-off using solid sodium dithionite. The output voltage is proportional to the oxygen concentration. At standard temperature and pressure, the amount of O_2 in 1 ml of air saturated water is 0.253 µmol/ml.

2.2.7. Mitochondrial respiration

Polarographic determination of oxidative phosphorylation was made by using oxygraph. The reaction mixture was taken into the water-jacketed reaction chamber, the reaction system contained 5 mM HEPES buffer, pH 7.2, 50 mM sucrose, 120 mM KCl, 5.55 mM $MgCl_2$ and freshly isolated mitochondria (200 mg protein) in a total reaction volume of 1.5 ml. After the addition of substrate (10 mM succinate), the rate of state III respiration was measured by the addition of 0.1 mM ADP and state IV respiration measured in absence of ADP (Gassner et al., 1997). Respiratory control index was determined as ratio of state III respiration to that of state IV. Phosphorylation efficiency was calculated as the ratio of amount of ADP used to that of amount of O_2 consumed during state III respiration.

2.2.8. Assay of respiratory enzyme complexes

Complex I activity was measured using ferricyanide as electron acceptor. The reaction system contained 250 mM sucrose, 50 mM potassium phosphate buffer, pH 7.2, 1 mM KCN, 5 mM $MgCl_2$, 1 mM potassium ferricyanide and 200 mg mitochondrial protein in a total volume of 1 ml. The reaction was started with 0.4 mM NADH and the rate of disappearance of either NADH (340 nm) or potassium ferricyanide (420 nm) was measured spectrophotometrically (Powel and Jackson, 2003). **Complex II** activity was measured using phenazinemethosulphate (PMS) as electron acceptor. The reaction mixture was same as used for complex I assay, except that ferricyanide was substituted with 1mM PMS and 70 µM 2,6-dichlorophenol indophenols (DCPIP). The rate of reduction of DCPIP was measured at 600 nm ($\epsilon_{\mu M}$ 16.2) (Powel and Jackson, 2003).

Complex IV reaction was measured in 2 ml reaction mixture, containing 60 µM reduced cytochrome c in 50 mM phosphate buffer, pH 7.2. The reaction was initiated by adding mitochondrial protein, and oxidation of cytochrome c was measured at 550 nm (Gassner et al., 1997). **F_0F_1 ATPase** activity was determined by quantifying the release of inorganic phosphate from ATP in 50 mM Tris-HCl, pH 7.4. The released phosphate was measured calorimetrically at 660 nm (Baginski et al., 1967).

2.2.9. Effect of insecticides on the mitochondrial respiration and respiratory enzyme complexes *in vivo*

Artificial diet was impregnated with 100 µM each of methylparathion, carbofuran, permethrin and fenvalarate, separately. Fourth-instar larvae were released individually in cups containing insecticide-impregnated diet. A group of larvae were kept as control. After 24 h, the mitochondria were isolated from the insecticide-fed larvae, and the isolated mitochondria evaluated for mitochondrial respiration and enzyme activities as described above.

2.2.10. Lipid peroxidation

Fourth-instar larvae were fed with different concentrations of methylparathion, carbofuran, permethrin, and fenvalarate (0 - 100 µM), and lipid peroxidation was measured by quantifying malondialdehyde (MDA) levels in larval homogenates on the basis of reaction with thiobarbituric acid to form a pink colored complex. To 0.5 ml of larval homogenates 3 ml of 1% phosphoric acid, 1 ml of 0.6% thiobarbituric acid was added, heated in close tube for 45 min and cooled. MDA produced was measured at 532 nm, and the nonspecific absorbance was subtracted by measuring the absorbance at 600 nm. Lipid peroxidation was calculated using 1.56 X 10^5 as extinction coefficient, and expressed as µmol of MDA/mg of protein extract (Poovala et al., 1997).

2.2.11. Lactate dehydrogenase leakage

Fourth-instar larvae were fed with different concentrations of methylparathion, carbofuran, permethrin, and fenvalarate (0 - 100 µM), and lactate dehydrogenase (LDH) activity was determined in larval homogenates in 2 ml reaction mixture containing 50 mM Tris-HCl, pH 7.4, 30 mM sodium pyruvate and 6.6 mM NADH, by measuring decrease in NADH content (ϵ_{mM} 6.32) at 340 nm by using UV spectrophotometer (Hitachi, U-2900). Enzyme activity was expressed as mmoles/min/mg protein (Poovala et al., 1997).

2.2.12. Measurement of H_2O_2 content

H_2O_2 content was estimated in larval homogenates according to Noreen and Ashraf (2009). Fourth-instar larvae were fed with different concentrations of methylparathion carbofuran, permethrin, and fenvalarate (0 - 100 µM). The larvae were homogenized in 0.1% trichloroacetic acid and centrifuged at 12,000 rpm for 15 min. To 0.5 ml of supernatant, 0.5 ml of phosphate buffer, 50 mM, pH 7.4, and 1 ml of 1 M

potassium iodide was added and absorbance was read at 390 nm and H_2O_2 content was calculated using extinction coefficient of 0.28 μM^{-1} cm^{-1} and expressed as µmoles of H_2O_2/mg protein.

2.2.13. Assay of antioxidant enzymes

Catalase and glutathione reductase (GR) were measured in insecticide-fed larvae. Catalase activity was determined by kinetic assay adapted from Olgun and Misra (2006). The 2 ml reaction mixture includes, 50 mM phosphate buffer, pH 7.4, 0.036% H_2O_2 and enzyme, in which the disappearance of peroxide is monitored spectrophotmetrically at 240 nm. One unit of catalase is equivalent to 1 µmol of H_2O_2 decomposed per minute per mg of protein using the extinction coefficient of 43.6 M^{-1} cm^{-1}. Glutathione reductase activity was determined in 1 ml reaction mixture, containing 1 ml 50 mM phosphate buffer, pH 7.2, 1 mM EDTA, 0.05% bovine serum albumin, 10 mM oxidized glutathione, and 10 mM NADPH. The rate of change in absorbance was measured at 340 nm. One unit of enzyme activity was expressed as 1 µmol of NADPH oxidized per minute per mg of protein (Olgun and Misra, 2006).

2.2.14. Bioassay of insecticides

Methylparathion, carbofuran, permethrin, and fenvalarate were incorporated into the artificial diet at different concentrations (0 to 100 µM). Artificial diet impregnated with insecticides was poured into small plastic cups (25 ml capacity) and the third-instar larvae were individually released into cups. The initial weights of *H. armigera* larvae were measured before releasing in the insecticide-impregnated artificial diets. The experiment was conducted in a completely randomized design (CRD) with three replications, and there were 10 larvae in each replication. The larval weights were recorded 5 days after initiating the experiment.

2.2.15. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) to judge the significance of differences between the treatments by using F-test, while the significance of differences between the treatment means was judged by least significant difference (LSD) at $p < 0.05$.

2.3. RESULTS

2.3.1. Mitochondrial respiration

Methylparathion, carbofuran, permethrin, and fenvalarate inhibited both the state III and state IV respiration in a dose-dependent manner when succinate was used as the oxidisable substrate. State III and state IV respiration for the control mitochondria was 63.24 ± 5.23 and 18.12 ± 2.13 nmoles/min/mg protein, respectively. At 100 µM concentration, methylparathion, carbofuran, permethrin and fenvalarate inhibited 79.23, 77.3, 65.13 and 72.54% state III respiration (Fig. 2.1 A), and 53.1, 45.92, 44.73 and 44.4% % state IV respiration, respectively (Fig. 2.1 B). Methylparathion and carbofuran also inhibited respiration control index (RCI) and P/O ratios in a dose-dependent manner, *in vitro*. The RCI and P/O ratio for the control mitochondria was 3.2 ± 0.41 and 2.21 ± 0.02, respectively, for succinate oxidation. The RCI was inhibited up to 55.32 and 58.44, 53.87, 58.43% by 100 µM methylparathion, carbofuran, permethrin and fenvalarate, respectively (Fig. 2.2 A), and the P/O ratio was inhibited 45.46 and 49.1%, respectively, by 100 µM methylparathion and carbofuran. Permethrin and fenvalarate did not affect the P/O ratios even at 100 µM (Fig. 2.2 B).

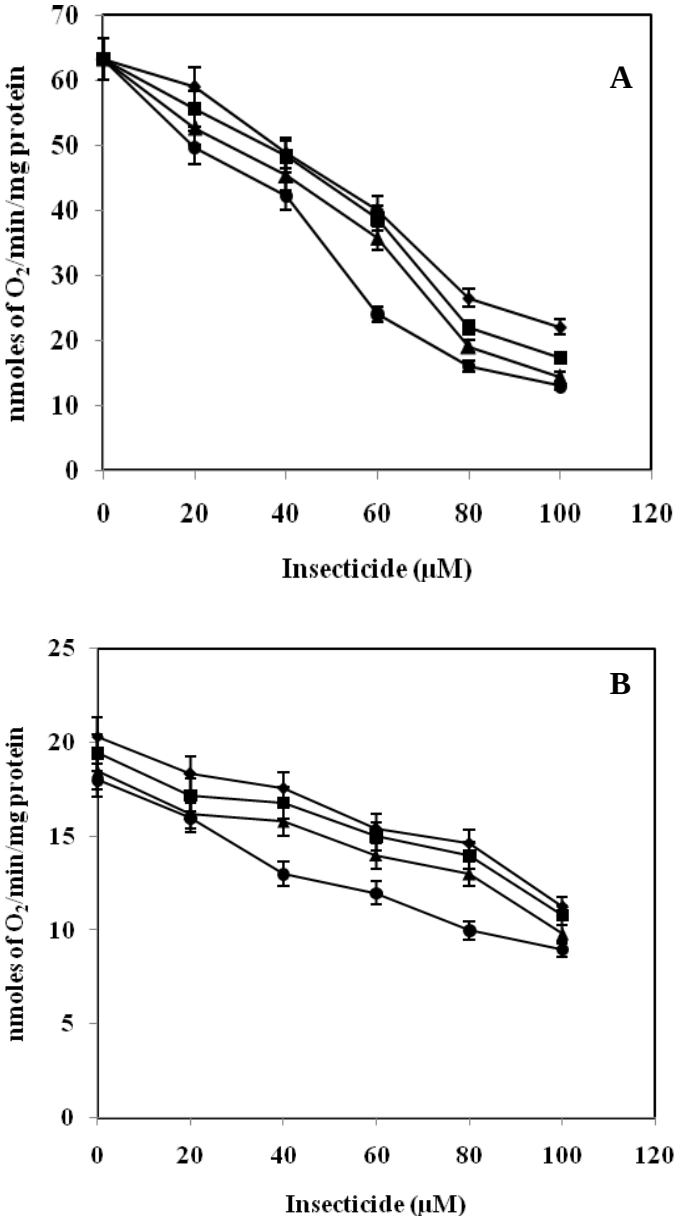


Fig. 2.1. Effect of methylparathion (●), carbofuran (▲), permethrin (♦) and fenvalarate (■) on mitochondrial respiration when succinate was the oxidisable substrate. Insecticides were incubated for 2 min with mitochondria, prior to addition of succinate. (A) Mitochondrial oxygen uptake in the presence of ADP (state III respiration), (B) mitochondrial oxygen uptake in absence of ADP (state IV respiration). The traces are the representative of three individual experiments.

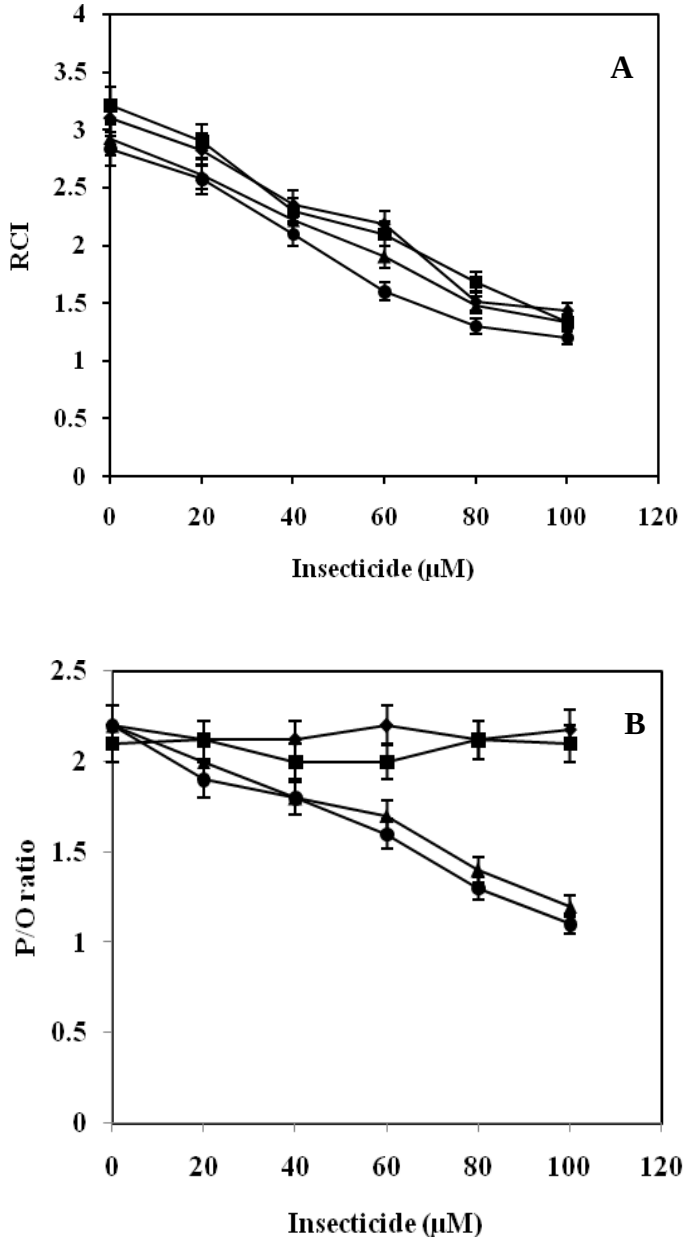


Fig. 2.2. Effect of methylparathion (●), carbofuran (▲), permethrin (♦) and fenvalarate (■) on RCI (A), and P/O ratio (B) when succinate was the oxidisable substrate. Insecticides were incubated for 2 min with mitochondria, prior to addition of succinate. The traces are the representative of three individual experiments.

2.3.2. Respiratory enzyme complexes

Mitochondrial respiratory enzyme complexes were measured in the presence of different concentrations of insecticides (0 to 100 µM). Among the enzyme complexes measured, methylparathion and carbofuran inhibited respiratory complexes II in a dose-dependent manner. At 100 µM, both the insecticides inhibited about 45 and 30% of the enzyme activities of complex II, respectively, with no significant effects on the activities of complex I, IV and F_0F_1 ATPase (Fig. 2.3 A,B). At 100 µM concentration, permethrin inhibited up to 70 and 22% of enzyme activities of complexes I and II, respectively (Fig. 2.4 A), whereas fenvalarate inhibited about 21% and 18% of enzyme complexes I and II, respectively, with no significant effect on the activities of complex IV and F_0F_1 ATPase (Fig. 2.4 B).

2.3.3. Effect of insecticides on mitochondrial respiration *in vivo*

Mitochondria isolated from insecticide-fed larvae exhibited a significant inhibition in state III and state IV respiration. The RCI was severely inhibited in case of methylparathion- and carbofuran-fed larvae *in vivo*. State III respiration was inhibited up to 47.0, 35.0, 44.98 and 50.59%, state IV respiration by 30.81, 20.17, 25.49 and 36.13%, RCI by 23.74, 18.76 26.16 and 22.64%, in larvae fed on diets containing methylparathion, carbofuran, permethrin, and fenvalarate, respectively (Fig. 2.5 A,B). P/O ratio was inhibited by 35.33 and 43.96% in methylparathion- and carbofuran-fed larvae, respectively (Fig. 2.5 A). None of the enzyme complexes were affected, but a drop of about 42% and 38% activity was observed for F_0F_1 ATPase in methylparathion- and carbofuran-fed larvae, respectively, as compared to the control larvae. P/O ratios and F_0F_1 ATPase were unaffected in permethrin- and fenvalarate-fed larvae (Fig. 2.5 B).

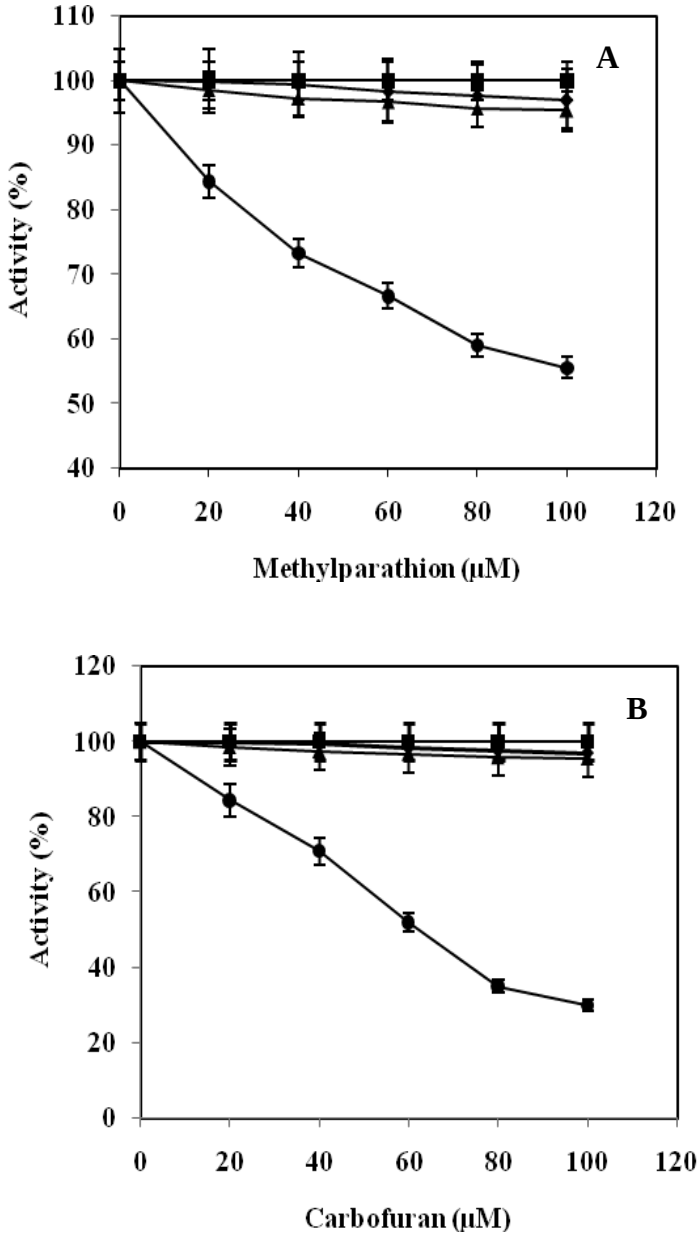


Fig. 2.3. Effect of methylparathion (A) and carbofuran (B) on NADH dehydrogenase (♦), succinate dehydrogenase (●), cytochrome oxidase (▲) and F_0F_1 ATPase (■) of isolated mitochondria. The data represents the Mean ± S.D. (n = 3). (Significantly different from control at * $p < 0.05$).

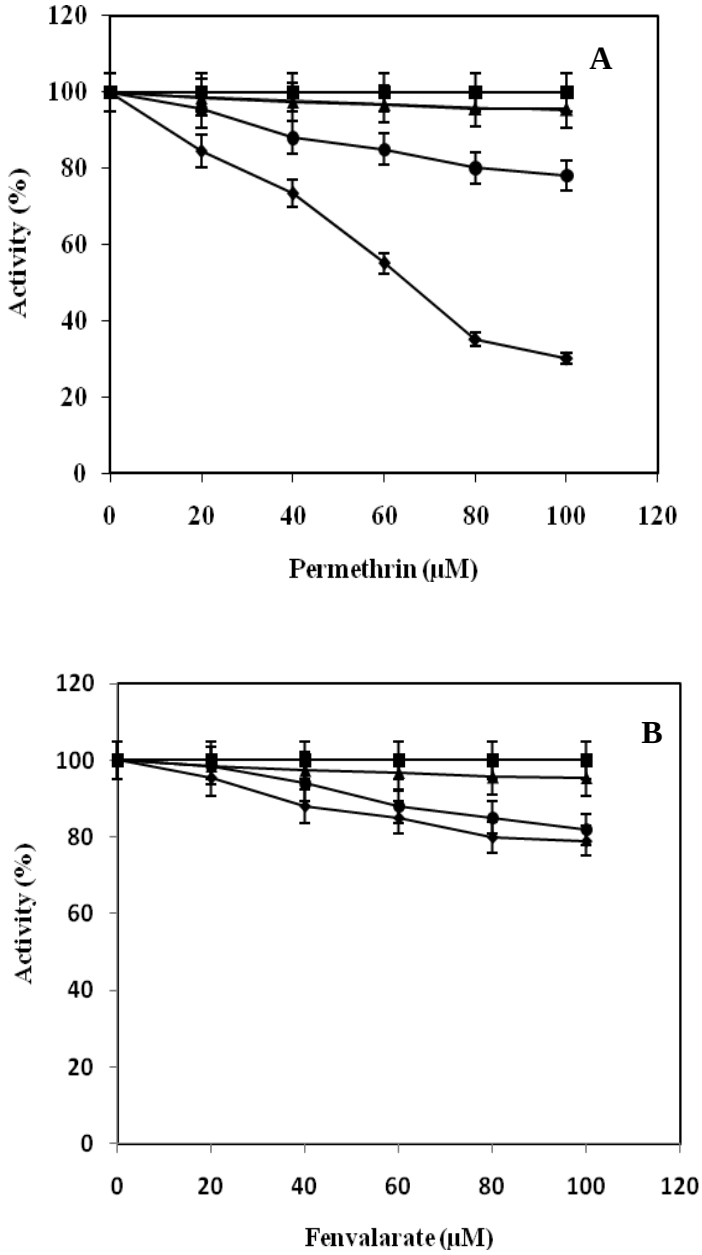


Fig. 2.4. Effect of permethrin (A) and fenvalarate (B) on NADH dehydrogenase (♦), succinate dehydrogenase (●), cytochrome oxidase (▲) and F_0F_1 ATPase (■) of isolated mitochondria. The data represents the Mean ± S.D. (n = 3).

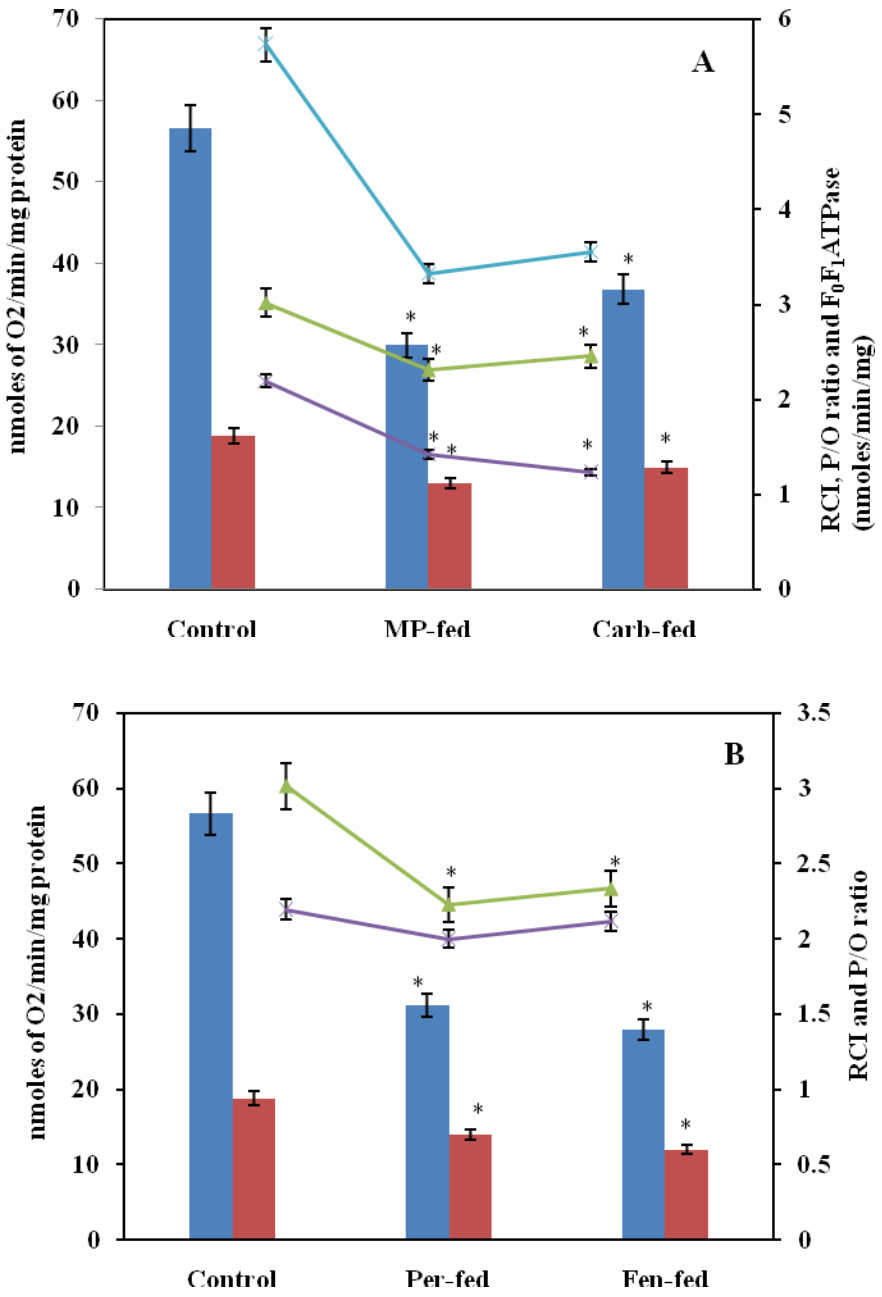


Fig. 2.5. *In vivo* effect of methylparathion, carbofuran (A), and permethrin and fenvalarate (B) on mitochondrial respiration for the oxidation of succinate. Oxygen consumption in presence of ADP (state III) (■), in absence of ADP (state IV) (■); RCI (■), P/O ratios (▲) and F_0F_1 ATPase activity (●) was measured in insecticide-fed larvae as described in text. The data represents the Mean ± S.D. (n = 3). (Significantly different from control at * $p < 0.05$).

2.3.4. Lipid peroxidation, lactate dehydrogenase leak and H_2O_2 content

In the control larvae fed on artificial diet without insecticides, lipid peroxidation was 0.5 mmoles/min/mg protein, H_2O_2 content 2.34 µmoles/min/mg protein, and LDH leak 0.116 µmoles/min/mg protein. There was proportional increase in lipid peroxidation (Fig. 2.6 A,B), LDH leak (Fig. 2.7 A,B) and H_2O_2 content (Fig. 2.8 A,B) in insecticide fed larvae. There was a significant increase in these components in the larvae fed on diets containing insecticides after 18 h of feeding. Maximum concentration was recorded at 24 h after feeding. For dose response studies, lipid peroxidation, LDH leak and H_2O_2 content were estimated after 24 h with 100 µM of methylparathion and carbofuran. There was a proportional increase in lipid peroxidation, LDH leak and H_2O_2 content in a time- and dose-dependent manner in larvae fed on diet containing methylparathion (105, 69, 149%), carbofuran (72, 64, 140%), permethrin (140, 201, 44%) and fenvalarate (90, 183, 61%), respectively.

2.3.5. Antioxidant enzymes

The activities of catalase and GR in control larvae were 11.74 ± 1.23 and 40.57 ± 2.64 U/mg, respectively. The activity of GR was reduced in a dose- and time-dependent manner, whereas, catalase was not affected in *H. armigera* larvae fed on insecticide containing diet (Tables 2.1, 2.2).

2.3.6. Biological effects of sublethal doses of insecticides on *H. armigera* larvae

All the insecticides inhibited the larval growth in a dose-dependent manner. At 100 µM, there was 64, 67, 64 and 70% inhibition in larval growth in larvae fed on diets with methylparathion, carbofuran, permethrin and fenvalarate, respectively (Fig. 2.9).

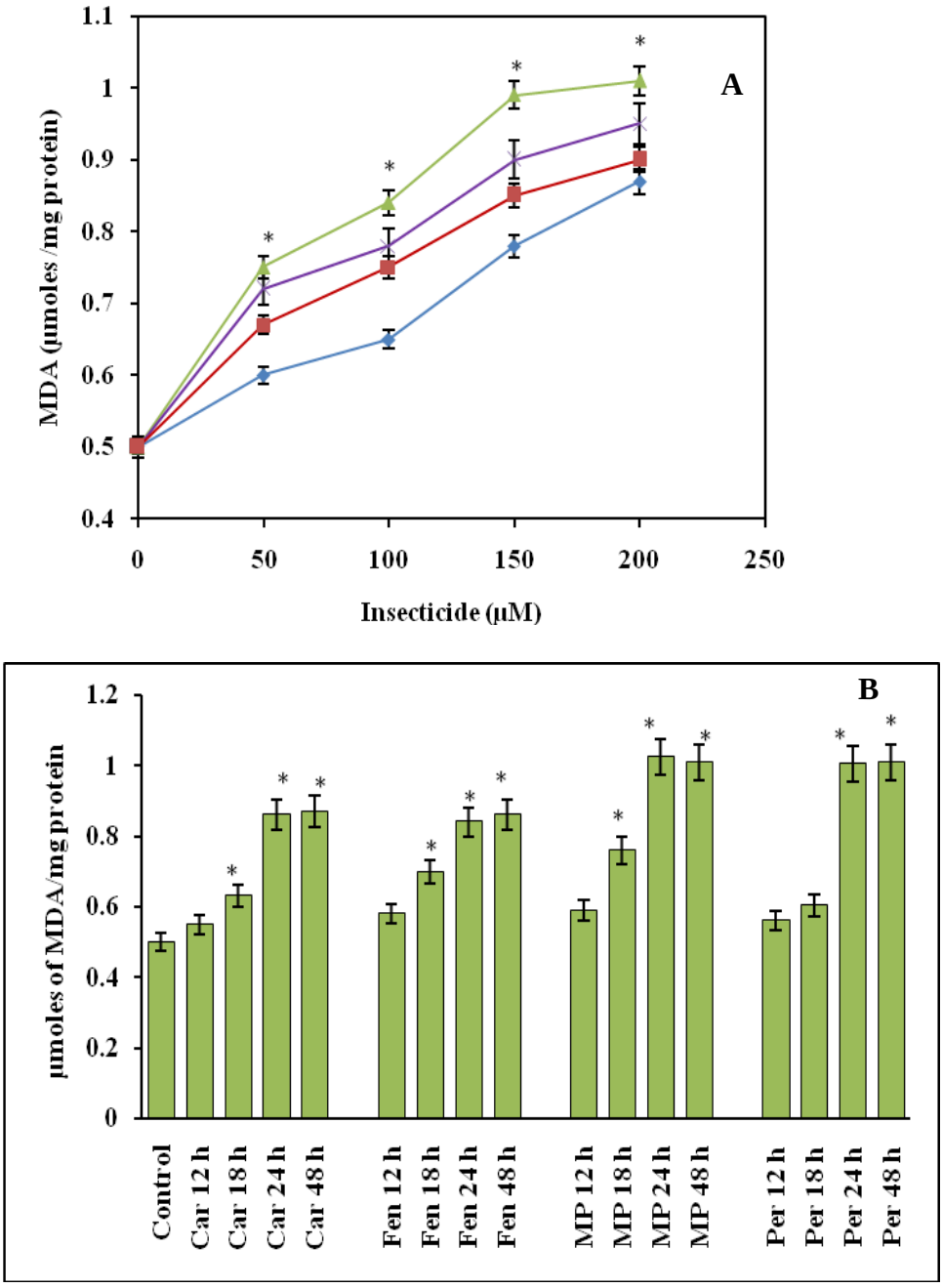


Fig. 2.6. (A) Dose-dependent response for lipid peroxidation in the larvae fed on diet containing methylparathion (▲), carbofuran (♦), permethrin (●) and fenvalarate (■). (B) Time-dependent response for lipid peroxidation at 12 h, 18 h, 24 h, and 48 h in insecticide-fed larvae. The data represents the Mean ± S.D. (n = 3). (Significantly different from control at * $p < 0.05$).

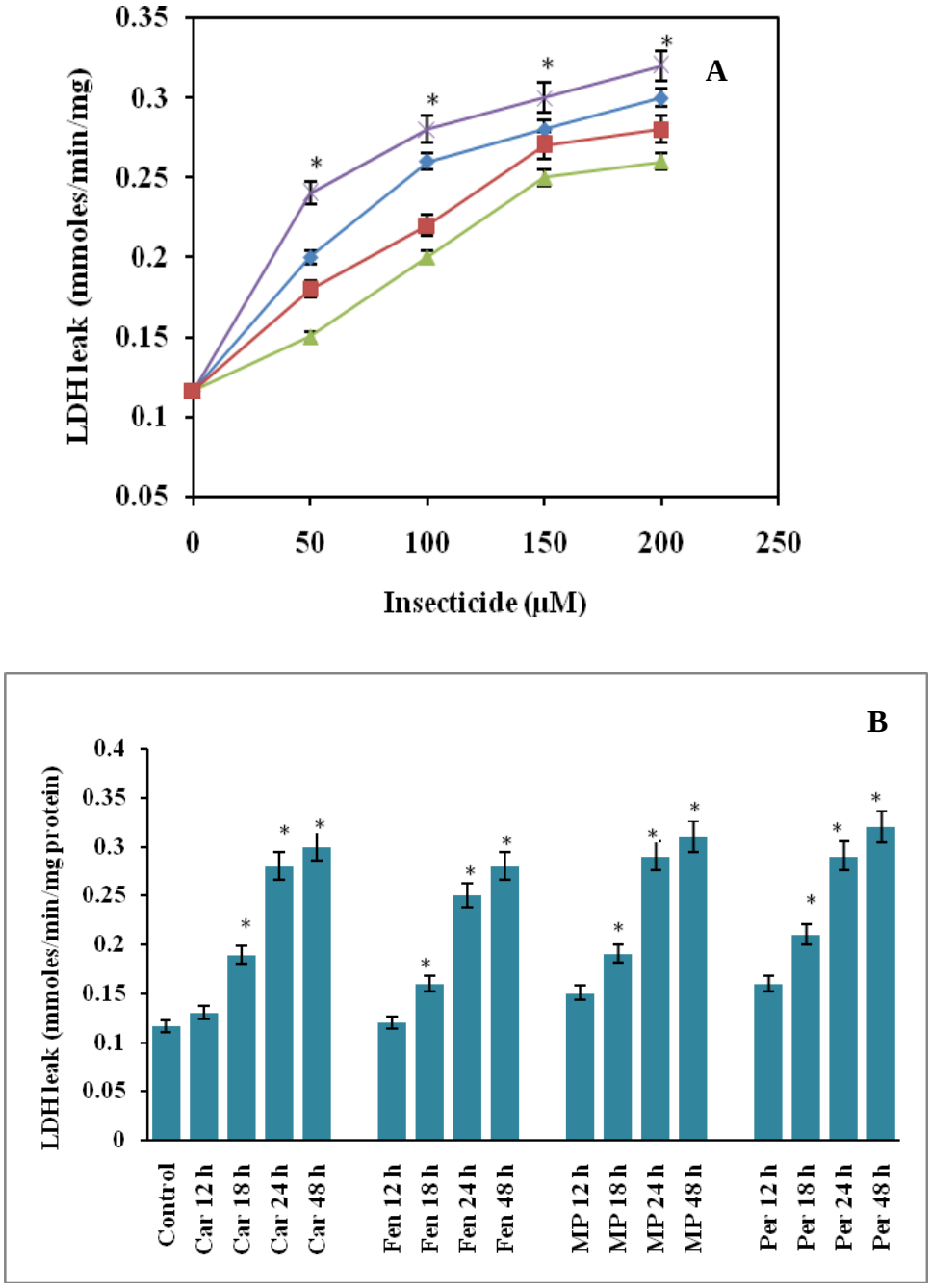


Fig. 2.7. (A) Dose-dependent response for LDH leak in the larvae fed on diet containing methylparathion (▲), carbofuran (♦), permethrin (●) and fenvalarate (■). (B) Time-dependent response for LDH leak at 12 h, 18 h, 24 h, and 48 h in insecticide-fed larvae. The data represents the Mean ± S.D. (n = 3). (Significantly different from control at * $p < 0.05$).

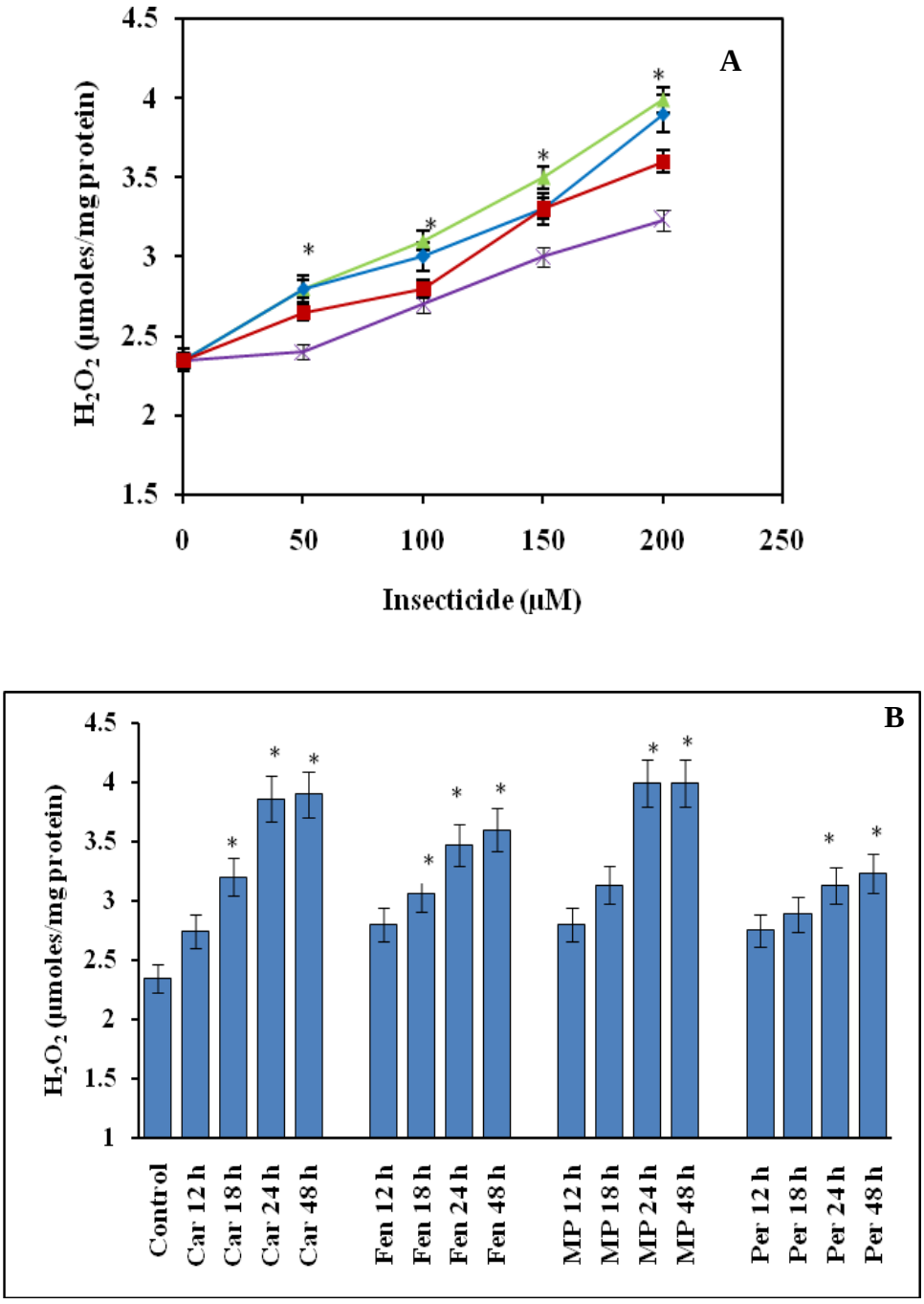


Fig. 2.8. (A) Dose-dependent response for H_2O_2 content in the larvae fed on diet containing methylparathion (▲), carbofuran (♦), permethrin (●) and fenvalarate (■). (B) Time-dependent response for H_2O_2 content at 12 h, 18 h, 24 h, and 48 h in insecticide-fed larvae. The data represents the Mean ± S.D. (n = 3). (Significantly different from control at * $p < 0.05$).

Table 2.1: Dose-dependent response of antioxidant enzymes in *H. armigera* fed on diet containing insecticide.

	0 µM	**20 µM**	**40 µM**	**60 µM**	**80 µM**	**100 µM**
MP-fed larvae						
Catalase (U/mg)	11.74 ± 1.23	11.82 ± 0.13	11.81 ± 1.45	11.91 ± 1.22	11.99 ± 0.22	12.24 ± 1.22
Glutathione reductase (U/mg)	40.57 ± 2.64	34.48 ± 2.63*	28.39 ± 3.22*	27.38 ± 1.73*	23.53 ± 2.73*	16.83 ± 1.82*
Carbofuran-fed larvae						
Catalase (U/mg)	11.74 ± 1.23	11.99 ± 0.23	11.98 ± 0.24	12.34 ± 0.34	12.44 ± 0.89	12.54 ± 1.11
Glutathione reductase (U/mg)	40.57 ± 2.64	32.45 ± 2.73*	26.37 ± 3.62*	23.12 ± 3.22*	20.28 ± 1.73*	19.87 ± 1.22*
Permethrin-fed larvae						
Catalase (U/mg)	11.74 ± 1.23	11.73 ± 0.89	11.74 ± 0.37	11.78 ± 0.83	11.82 ± 1.34	12.39 ± 1.34
Glutathione reductase (U/mg)	40.57 ± 2.64	36.51 ± 1.22*	28.39 ± 3.82*	25.31 ± 2.82*	22.32 ± 1.83*	15.01 ± 2.73*
Fenvalarate-fed larvae						
Catalase (U/mg)	11.74 ± 1.23	11.64 ± 0.99	11.34 ± 0.98	12.34 ± 1.34	12.44 ± 1.11	12.84 ± 1.12
Glutathione reductase (U/mg)	40.57 ± 2.64	34.89 ± 2.73*	27.38 ± 2.82*	23.93 ± 1.22*	20.08 ± 1.22*	18.66 ± 2.11*

Values in the Table represents Mean ± S.D. of at least three determinants (significantly different from control at * $p < 0.05$).

Table 2.2: Time-dependent response of antioxidant enzymes in *H. armigera* fed on diet containing insecticide.

	Control	**12 h**	**18 h**	**24 h**	**48 h**
MP-fed larvae					
Catalase (U/mg)	11.74 ± 1.23	11.76 ± 0.93	11.92 ± 0.64	12.24 ± 1.22	12.28 ± 0.83
Glutathione reductase (U/mg)	40.57 ± 2.64	32.88 ± 2.28*	26.64 ± 3.22*	16.83 ± 1.82*	17.11 ± 2.63*
Carbofuran-fed larvae					
Catalase (U/mg)	11.74 ± 1.23	11.82 ± 1.02	12.11 ± 1.45	12.54 ± 1.11	12.66 ± 1.54
Glutathione reductase (U/mg)	40.57 ± 2.64	34.98 ± 1.98	27.44 ± 2.43*	19.87 ± 1.22*	19.88 ± 3.74*
Permethrin-fed larvae					
Catalase (U/mg)	11.74 ± 1.23	11.99 ± 0.85	12.12 ± 0.99	12.39 ± 1.34	12.44 ± 1.11
Glutathione reductase (U/mg)	40.57 ± 2.64	36.49 ± 3.28	22.63 ± 2.73*	15.01 ± 2.73*	16.73 ± 1.22*
Fenvalarate-fed larvae					
Catalase (U/mg)	11.74 ± 1.23	11.82 ± 1.87	12.45 ± 0.54	12.84 ± 1.12	12.92 ± 1.34
Glutathione reductase (U/mg)	40.57 ± 2.64	34.96 ± 2.87*	29.43 ± 1.82*	18.66 ± 2.11*	19.45 ± 2.63*

Values in the Table represents Mean ± S.D. of at least three determinants (significantly different from control at * $p < 0.05$).

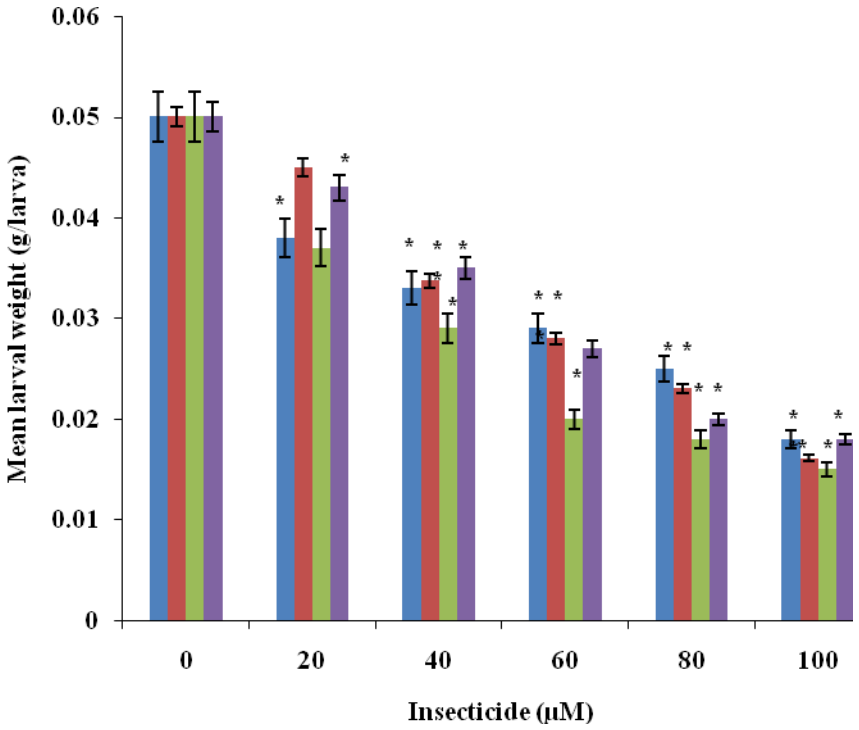


Fig. 2.9. Bioassays for insecticides. Neonates were fed on artificial diet containing varying concentrations (1 to 100 µM) of methylparathion, carbofuran, permethrin and fenvalarate. The data represents the Mean ± S.D. (n = 3) (Significantly different from control at * $p < 0.05$). [Methylparathion (Blue), carbofuran (pink), permethrin (green), and fenvalarate (violet)].

2.4. DISCUSSION

Organophosphate and carbamate insecticides inactivate acetylcholine esterase, inhibiting the breakdown of acetylcholine, leading to accumulation of acetylcholine, which initially over-stimulates and then paralyzes the cholinergic transmission. Phosphorylation and carbamoyaltion of acetylcholine esterase is the major difference in mode of action of organophosphates and carbamates, respectively. Pyrethroids are toxic to the central nervous system of both insects and mammals (Husain et al., 1994) with its primary target including voltage-dependent sodium channel (Soderlund, 1985). Pyrethroids being hydrophobic could effects on biological membranes at sites other than voltage-dependent sodium channel (Chinn and Narahashi, 1986). Mitochondria are also primary sub-cellular target for insecticide, many of them inhibit the enzymatic activity and alter mitochondrial functions (Carlson and Ehrich, 1999). Mitochondrial metabolism is responsible not only for the generation of energy, but also for free radicals (Beal, 1998). Some studies suggested that the exposure to a wide range of pesticides could result in the production of free radical and the inactivation of components of the mitochondrial respiratory chain (Turner et al., 2003). Hence, in the present studies, effects of methylparathion, carbofuran, permethrin and fenvalarate were studied on the energy metabolism in mitochondria, respiratory enzyme activities and oxidative stress to understand the effects of these insecticides in insect system.

ADP-stimulated respiration (state III) was inhibited by all the insecticides *in vitro* when succinate was used as the oxidizing substrate. The insecticides also inhibited the rate of oxygen consumption after ADP exhaustion (state IV). Both, state III and state IV respiration were inhibited in dose-dependent manner (Fig. 2.1 A,B). RCI and P/O ratios

were also inhibited in a dose-dependent manner in isolated mitochondria with an exception of pyrethroids on P/O ratio (Fig. 2.2 A,B). A clear inhibitory effect on succinate dehydrogenase is induced by methylparathion and carbofuran (Fig. 2.3 A,B), whereas permethrin and fenvalarate inhibited both complexes I and II *in vitro* (Fig. 2.4 A,B), suggesting the inhibition of electron flow through the electron transport chain. Thus the insecticide-induced depression of phosphorylation efficiency of isolated mitochondria was mainly due to the inhibition of oxygen consumption and inhibition at respiratory enzyme complex level in case of methylparathion and carbofuran. Similar observations were reported for the insecticide-induced mitochondrial dysfunction *in vitro* in rats (Moreno and Madeira, 1990).

Under *in vivo* conditions, state III respiration and state IV respiration decreased, affecting RCI, suggesting absence of uncoupling mechanism of action in these insecticides (Fig. 2.5). Mitochondria prepared from control larvae had a RCI of 3.01 whereas the mitochondria from methylparathion-, carbofuran-, permethrin- and fenvalarate-fed larvae had a RCI of about 1.66, 1.67, 2.22, and 2.33 respectively. The decrease in RCI further confirmed that these insecticides result in mitochondrial injury, *in vivo*. Phosphorylation efficiency (P/O) of the mitochondria was inhibited in larvae fed on diets containing methylparathion and carbofuran, which is due to inhibition in the activity of F_0F_1 ATPase (Fig. 2.5 A). Whereas, the phosphorylation efficiency and the activity of F_0F_1 ATPase was unaffected in permethrin- and fenvalarate-fed larvae (Fig. 2.5 B). Reduced rate of phosphorylation observed *in vitro* with methylparathion in rat liver mitochondria is due to inhibition of phosphate carrier and dislocation of F_0 and F_1 components of ATP synthase, which leads to less ATP content (Moreno and Madeira,

1990). Chlorpropham, a carabamate insecticide, is also results in ATP depletion in mitochondria (Nakagawa et al., 2004). Since mitochondrial respiratory chain produces the majority of ATP content of the cells, an impairment in the mitochondrial function could adversely affect the energetic state of the cell.

There are several pathways by which pesticides are thought to induce oxidative stress. Pesticides inhibit the mitochondrial electron-transfer chain reaction, leading to accumulation of semi ubiquitous, which enables it to transfer one electron to molecular oxygen to form superoxide radicals (Wang et al., 2004). Further, it may also interference with cellular antioxidant defense system via alteration in activities of antioxidant enzymes *viz.* catalase and status of glutathione (Sandrini et al., 2006). Oxygen free radicals and hydroperoxides are collectively termed reactive oxygen species (ROS). Chemical toxic pollutants are important sources of ROS in biological systems (Bacchetta, 2011). ROS are formed continuously in cells as a consequence of both oxidative biochemical reactions and external factors. However, they become harmful when they are produced in excess in certain abnormal conditions such as exposure to certain environment pollutants. Under these conditions, the endogenous antioxidants may be unable to encounter ROS formation and the ROS formed may cause cellular damage by peroxidation of membrane lipids, inactivation of enzymes, cross-linking and breakdown of DNA. The damage may be involved in the etiology of diverse pathological conditions such as neurodegeneration (Olgun and Misra, 2006). Lipid peroxidation, H_2O_2 content and LDH leak were measured as oxidative stress markers; in our results, a time- and dose-dependent increase in these components was recorded in insecticide-fed larvae (Fig. 2.6 - 2.8). There was a little increase in oxidative stress markers after 12 h of

feeding on diets containing the insecticides, but a significant increase was recorded after 18 h, reaching the maximum level at 24 h. Highly reactive oxygen metabolites, especially hydroxyl radicals, act on unsaturated fatty acids of phospholipid components of membranes to produce malondialdehyde, a lipid peroxidation product. A significant increase in the MDA content in insecticide-fed larvae was observed (Fig. 2.6). Lipid peroxidation has been suggested as one of the molecular mechanisms involved in organophosphates, carabamtes, organochlorines and pyrethroids. Chlorpyrifos have been reported to induce oxidative stress, with enhanced MDA production (Goel et al., 2005). These findings are similar to earlier reports, wherein, toxicity of many xenobiotics, including pesticides has been found to be associated with the generation of ROS (Sohn et al., 2004). The LDH activity is the most sensitive parameter for evaluation of tissue damage and toxicity. Significant increase in LDH activity in insecticide-fed larvae (Fig. 2.7) indicated the higher rates of glycolysis, indicating that aerobic oxidation was adversely affected in insecticide-fed larvae, as confirmed by inhibition in oxygen uptake *in vivo*. Elevated levels of LDH activity have been associated with inhibition of aerobic oxidation in pesticide exposed fish (Ghosh, 1989). Bidrin, an organophosphate insecticide, induced lipid peroxidation, H_2O_2 and LDH levels in cultured renal tubular cells (Poovala et al., 1999), while carbofuran-induced mitochondrial dysfunction and lipid peroxidation in rat brain (Kamboj et al., 2008).

The antioxidant enzymes constitute the first line of defense against oxidative stress. Pesticides are known to alter the level of antioxidant enzymes. In insecticide-fed *H. armigera* larvae, catalase activity was unaffected whereas glutathione reductase was inhibited in a dose- and time-dependent manner (Table 2.1, 2.2). Similar observations

have earlier been made by which Olgun and Misra (2006). However, lindane, an organochlorine insecticide, reduced the activity of liver catalase, but did not affect glutathione reductase (Janqueira et al., 1986). Catalase is ubiquitously present in a wide range of aerobic cell types, known to degrade H_2O_2 into H_2O and O_2. Because the K_m value for the catalysis of H_2O_2 by catalase is in the range of 1.1 M, and the increased levels of H_2O_2 produced during exposure to insecticides never exceeded this level, no change in levels of catalase are not surprising. Reduction in glutathione reductase levels may be because of a direct effect of these insecticides and their metabolites on this enzyme. However, the antioxidant enzymes SOD, GST and CAT limit the effects of oxidant molecules on tissues and are active in the defense against oxidative cell injury by means of their being free radical scavengers (Jalaili et al., 2007). These enzymes work together to eliminate active oxygen species and small deviations in physiological concentrations may have a dramatic effect on the resistance of cellular lipids, proteins and DNA to oxidative damage.

Bioassays carried out with insecticides shown a dose-dependent response on larval growth (Fig. 2.9). Reduction in larval growth in larvae fed on diets with these insecticides may be due to impairment in the mitochondrial function as evidenced by depression in mitochondrial respiration, respiratory control index (RCI), P/O ratio and increase in oxidative stress as evidenced by high levels of lipid peroxidation, H_2O_2 content, and LDH leak, under *in vivo* conditions. OPI-induced oxidative stress is associated with the degeneration of neurons and apoptosis (Kaur et al., 2007).

In conclusion, insecticide exposure impedes mitochondrial respiratory functions and induced lipid peroxidation, H_2O_2 content and LDH leak in a time- and dose-dependent manner, altered the level of antioxidant enzymes, leading to oxidative stress in cells, resulting in deleterious effects on the growth of *H. armigera* larvae, along with the neurotoxic effects. There were some differences in mode of action of these chemicals in mitochondrial oxidation, and hence, these can be used alternatively for the control of *H. armigera*.

Chapter III

Interaction of salicylic acid and jasmonic acid on the mitochondria of Helicoverpa armigera

3.1. INTRODUCTION

Plants are continuously exposed to the challenge of a variety of herbivores. However, to defend themselves against the herbivores, plants have evolved constitutive and inducible defense mechanisms. Salicylic acid (SA) and jasmonic acid (JA) function as signaling molecules in plants, mediating induced plant responses against herbivores and pathogen infection, leading to the activation of genes involved in host plant resistance to insects (Arimura et al., 2005). Both, SA and JA are involved in plants' induced defense response, and both cause systemic acquired resistance (SAR) to pathogens and mechanical injury in plants (Cohen and Flescher, 2009). SA is involved in regulating plant metabolism, and local and endemic disease resistance in plants in response to pathogen infection (Alverez, 2000). More recently, elevated levels of SA have been found to be associated with tobacco mosaic infection and herbivore attack in tobacco (Malamy et al., 1990) and tomato (Peng et al., 2004) plants, respectively. JA causes the induction of proteinase inhibitors (Farmer and Ryan, 1990) and also raises the ROS level in plants, which confers resistance to insect pests. JA is produced quickly following insect feeding (Bi et al., 1997).

SA and its analogues have shown adverse effects on oxidative phosphorylation and swelling in rat liver mitochondria (Battaglia et al., 2005). It also induces the mitochondrial permeability transition (MPT) in the presence of calcium in isolated liver mitochondria (Biban et al., 1995). Recent studies have shown that jasmonates can also suppress proliferation of human cancer cells, and one of the probable reasons may be mitochondrial dysfunction, consisting of mitochondrial membrane permeability transition, dissipation of inner membrane potential, osmotic swelling of the matrix and

release of cytochrome c (Rotem et al., 2005). Since the level of these signaling molecules increases during insect and pathogen attack, the present studies were therefore undertaken to determine whether SA and JA could have a direct effect on the growth of *H. armigera.*

3.2. MATERIALS AND METHODS

3.2.1. Glassware cleaning

Glassware cleaning procedure was similar to those as described in chapter II under the section 2.2.1.

3.2.2. Chemicals

NADH, bovine serum albumin (BSA), ADP, jasmonic acid, salicylic acid and cyclosporine A were purchased from Sigma Aldrich (Mumbai, India). Sucrose, pyruvate, and malate were purchased from Qualigens (Mumbai, India), while the other chemicals used were of analytical grade.

3.2.3. Insect culture

Larvae of *H. armigera* were obtained from the insect rearing laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. The larvae were reared on a chickpea based semi-synthetic diet under laboratory conditions at 27 ± 1 ˚C, 65 ± 5% RH, and 12 h photoperiod (Armes et al., 1992). Preparation of artificial diet and rearing of *H. armigera* larvae is described in chapter II under the section 2.2.3.

3.2.4. Isolation of mitochondria

H. armigera larvae were starved for 3 h for the isolation of mitochondria. The mitochondria were isolated from the fourth- and fifth-instar larvae, and the experimental details are given in chapter II under the section 2.2.4.

3.2.5. Protein estimation

Protein concentration was determined by Lowry's method (1951) using BSA as a standard.

3.2.6. Mitochondrial respiration

Polarography determination of oxidative phosphorylation was made in oxygraph fitted with a Clark type oxygen electrode. Respiratory parameters were measured in presence or absence of SA or JA for the substrates [NADH (1 mM) or succinate (10 mM) or pyruvate plus malate (5 mM)]. The experimental details were described in chapter II under the section 2.2.7.

3.2.7. Enzyme assays

The respiratory enzyme complexes were assayed in presence of varying concentrations of SA and JA (0 to 2 mM). Assay procedure for the enzyme complexes I, II, IV and F_0F_1 ATPase were described in chapter II under the section 2.2.8.

3.2.8. Mitochondrial swelling

The mitochondrial swelling in isolated mitochondria was determined as a function of time by monitoring the absorbance changes at 540 nm in the standard reaction mixture used for polarographic studies in presence of 0.1 mM Ca^{2+}. Succinate was used as the oxidizable substrate (Battaglia et al., 2005).

3.2.9. Detection of cytochrome c release

Mitochondria (1 mg protein) were incubated in 50 mM phosphate buffer, pH 7.2, containing varying concentrations of SA and JA at different time intervals in the presence of 0.1 mM Ca^{2+}. The reaction mixtures were centrifuged at 12,000 x g for 10 min at 4 ˚C to obtain mitochondrial pellets. The supernatants were further spun at 12,000 x g for 1 h

to eliminate mitochondrial fragments. The supernatants were collected and the released cytochrome c was estimated according to Chamberlin (2004).

3.2.10. Effect of SA and JA on the mitochondrial respiration and respiratory enzyme complexes *in vivo*

Fourth-instar larvae were fed on artificial diet containing 0.5 mM SA and 0.5 mM JA separately. After 24 h, mitochondria were isolated from SA- and JA-fed larvae, and the isolated mitochondria evaluated for mitochondrial respiration and enzyme activities as described in chapter II, sections, 2.2.7. and 2.2.8.

3.2.11. ATP content in SA- and JA-fed larvae

Fourth-instar larvae were fed on artificial diet containing 0.5 mM SA and 0.5 mM JA separately. After 24 h, ATP content was estimated in their post-mitochondrial supernatant of larval extract by HPLC, according to the method of Jones (1981).

3.2.12. Lipid peroxidation

Fourth-instar larvae were fed with different concentrations of SA and JA (0 to 2 mM), and lipid peroxidation was measured by quantifying malondialdehyde level in larval homogenates as described earlier in chapter II, section 2.2.10.

3.2.13. Lactate dehydrogenase leakage

Fourth-instar larvae were fed with different concentrations of SA and JA (0 to 2 mM), and lactate dehydrogenase (LDH) activity was determined in larval homogenates. The assay procedure was described in chapter II, section 2.2.11.

3.2.14. Measurement of H_2O_2 content

Fourth-instar larvae were fed with different concentrations of SA and JA (0 to 2 mM). H_2O_2 content was measured according the procedure described in chapter II, section 2.2.12.

3.2.15. Bioassay of SA and JA

SA and JA were incorporated into the artificial diet at different concentrations (0 to 2 mM) was poured into small plastic cups (25 ml capacity). Bioassays were carried out with SA/JA-impregnated diet with *H. armigera* neonates according to the experimental details mentioned in chapter II, section 2.2.14.

3.2.16. Statistical analysis

Data were subjected to one way analysis of variance (ANOVA) as described in chapter II, section 2.2.15.

3.3. RESULTS

3.3.1. The effect of SA and JA on mitochondrial respiration *in vitro*

Fig. 3.1. shows the effect of SA and JA on the oxidation of succinate in intact mitochondria, with RCI and P/O ratios. SA inhibited the ADP-stimulated respiration (state III) in a dose-dependent manner. At 2 mM, SA inhibited state III respiration by 64%, 62%, and 58% for the oxidation of NADH, succinate, and pyruvate plus malate, respectively. The oxygen consumption was stimulated at lower concentrations (<1 mM) but declined at higher concentrations (above 1 mM). Similarly at 2 mM, it inhibited state IV respiration by 30%, 28%, and 20%, when NADH, succinate, and pyruvate plus malate were the substrates, respectively. Respiratory control index (RCI) decreased with an increase in SA concentration. The RCI was decreased by 50%, 31%, and 53% by 2 mM

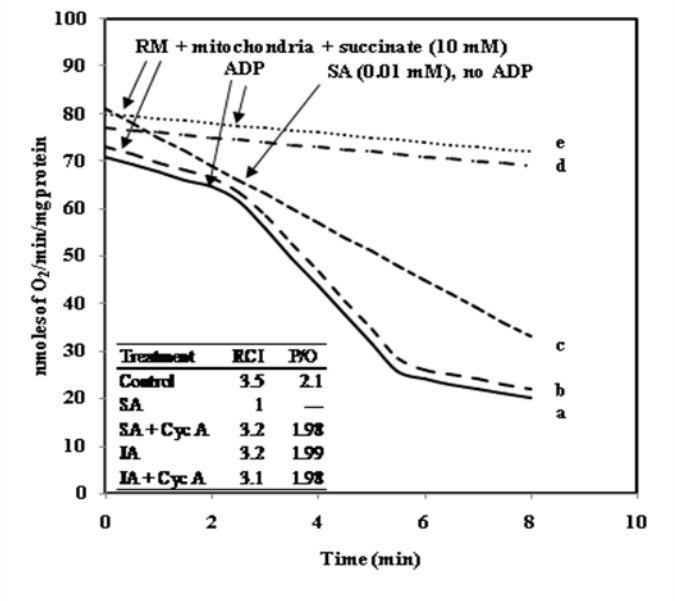


Fig. 3.1. Oxidation of succinate in isolated mitochondria. Oxygen consumption was measured using oxygen electrode for the oxidation of succinate in isolated mitochondria in absence of the signaling molecules (control) (a), in presence of 2 mM JA (b), in presence of 0.01 mM SA (state IV respiration) (c), in presence of 2 mM SA (state III respiration) (d), and in presence of 1 mM KCN (e). In the corner, a table is shown indicating the RCI and P/O ratios.

SA, when NADH, succinate, and pyruvate plus malate were the substrates, respectively (Table 3.1). However, JA did not show any effect on the oxidative phosphorylation and RCI of the isolated mitochondria with any of the substrates (Table 3.2).

3.3.2. Enzyme assays

Mitochondrial respiratory enzyme complexes were measured in the presence of different concentrations of SA and JA (0 to 2 mM). Among the enzyme complexes measured, SA inhibited the respiratory complexes I and II in a dose-dependent manner. At 2 mM concentration, it inhibited 30% and 40% of the enzyme activities, respectively. No significant effect of SA was observed on the activities of complex IV and F_0F_1 ATPase (Fig. 3.2 A). In contrast, JA had no effect on any of the respiratory enzyme complexes, even at higher concentrations (Fig. 3.2 B).

3.3.3. Mitochondrial swelling

Mitochondria incubated with 0.5 mM SA in standard reaction mixture in the presence of calcium exhibited extensive swelling, as evidenced by a strong decrease in the apparent absorbance of the mitochondrial suspension. However, pre-treatment with cyclosporine A (50 µM), an inhibitor of membrane permeability transition (MPT) pore, greatly inhibited the decrease in absorbance, indicating that SA-induced mitochondrial swelling depends on the opening of the MPT. However, JA did not induce mitochondrial swelling (Fig. 3.3).

3.3.4. Cytochrome c release

SA induced the efflux of cytochrome c in a time- and dose-dependent manner when incubated with isolated mitochondria in the presence of Ca^{2+}. When mitochondria (about 1 mg protein) was incubated with SA (2 mM), 1.76 nmol of cytochrome c released

Table 3.1: In vitro effect of SA on oxidation of succinate, NADH, and pyruvate plus malate in mitochondria of *H. armigera*.

	Succinate oxidation			NADH oxidation			Pyruvate and malate oxidation		
SA (mM)	nmoles of O_2 consumed / min/mg of protein			nmoles of O_2 consumed / min/mg of protein			nmoles of O_2 consumed / min/mg of protein		
	State III	State IV	RCI	State III	State IV	RCI	State III	State IV	RCI
0	57.23 ± 2.89	19.22 ± 0.61	2.98 ± 0.12	77.96 ± 1.32	27.49 ± 0.31	2.83 ± 0.04	63.33 ± 0.94	20.58 ± 1.28	3.07 ± 0.14
0.1	53.72 ± 1.70*	22.09 ± 0.46	2.43 ± 0.05*	73.61 ± 0.86*	29.33 ± 0.73	2.51 ± 0.07	61.43 ± 0.63*	22.3 ± 0.76	2.77 ± 0.14*
0.5	51.26 ± 1.04*	24.82 ± 0.80	2.07 ± 0.03*	71.08 ± 0.72*	31.85 ± 0.63	2.23 ± 0.04	58.89 ± 0.44*	25.9 ± 0.76	2.28 ± 0.07*
1.0	51.18 ± 0.93*	19.14 ± 0.68	2.68 ± 0.14*	59.03 ± 0.76*	27.24 ± 1.09	2.16 ± 0.05	40.7 ± 0.51*	18.83 ± 0.89	2.16 ± 0.08*
1.5	42.64 ± 0.76*	18.06 ± 0.32	2.36 ± 0.01*	33.05 ± 0.84*	23.15 ± 0.61*	1.42 ± 0.007*	27.5 ± 1.77*	17.64 ± 0.17	1.55 ± 0.19*
2.0	28.12 ± 1.63*	13.81 ± 0.98*	2.03 ± 0.28*	27.86 ± 1.34*	19.10 ± 0.66*	1.41 ± 0.07*	23.79 ± 1.51*	16.55 ± 1.63	1.44 ± 0.10*

Values are mean ± SD (n = 3).

* Significantly different at $p < 0.05$ as compared to the control (no SA).

Table 3.2: *In vitro* effect of JA on oxidation of succinate, NADH, and pyruvate plus malate in mitochondria of *H. armigera.*

	Succinate oxidation			NADH oxidation			Pyruvate plus malate oxidation		
	nmoles of O_2 consumed/min/mg protein			nmoles of O_2 consumed/min/mg protein			nmoles of O_2 consumed/min/mg protein		
JA (mM)	**State III**	**State IV**	**RCI**	**State III**	**State IV**	**RCI**	**State III**	**State IV**	**RCI**
0	109.6 ± 3.90	34.44 ± 1.14	3.18 ± 0.17	80.23 ± 4.56	26.67 ± 4.57	3.01 ± 0.48	88.48 ± 8.53	28.23 ± 4.06	3.13 ± 0.36
0.1	108.42 ± 5.14	34.41 ± 1.60	3.15 ± 0.18	78.65 ± 6.04	26.55 ± 4.16	2.96 ± 0.65	85.44 ± 6.53	28.05 ± 1.13	3.05 ± 0.11
0.5	105.76 ± 9.89	33.85 ± 8.49	3.12 ± 1.31	77.54 ± 7.07	26.54 ± 5.74	2.92 ± 0.59	84.88 ± 5.85	27.79 ± 0.78	3.05 ± 0.27
1.0	104.42 ± 2.26	33.77 ± 8.53	3.09 ± 0.32	77.87 ± 9.06	25.89 ± 3.18	3.01 ± 0.49	84.51 ± 8.04	27.65 ± 0.53	3.06 ± 0.35
1.5	99.36 ± 3.40	32.23 ± 0.57	3.08 ± 0.14	76.89 ± 4.72	25.77 ± 5.07	2.98 ± 0.31	84.48 ± 4.06	27.59 ± 2.01	3.06 ± 0.22
2.0	95.02 ± 0.47	31.58 ± 1.50	3.01 ± 0.16	75.5 ± 7.14	25.56 ± 2.78	2.95 ± 0.07	82.25 ± 5.24	26.89 ± 1.69	3.06 ± 0.40

Values are representation of mean ± SD (n = 3).

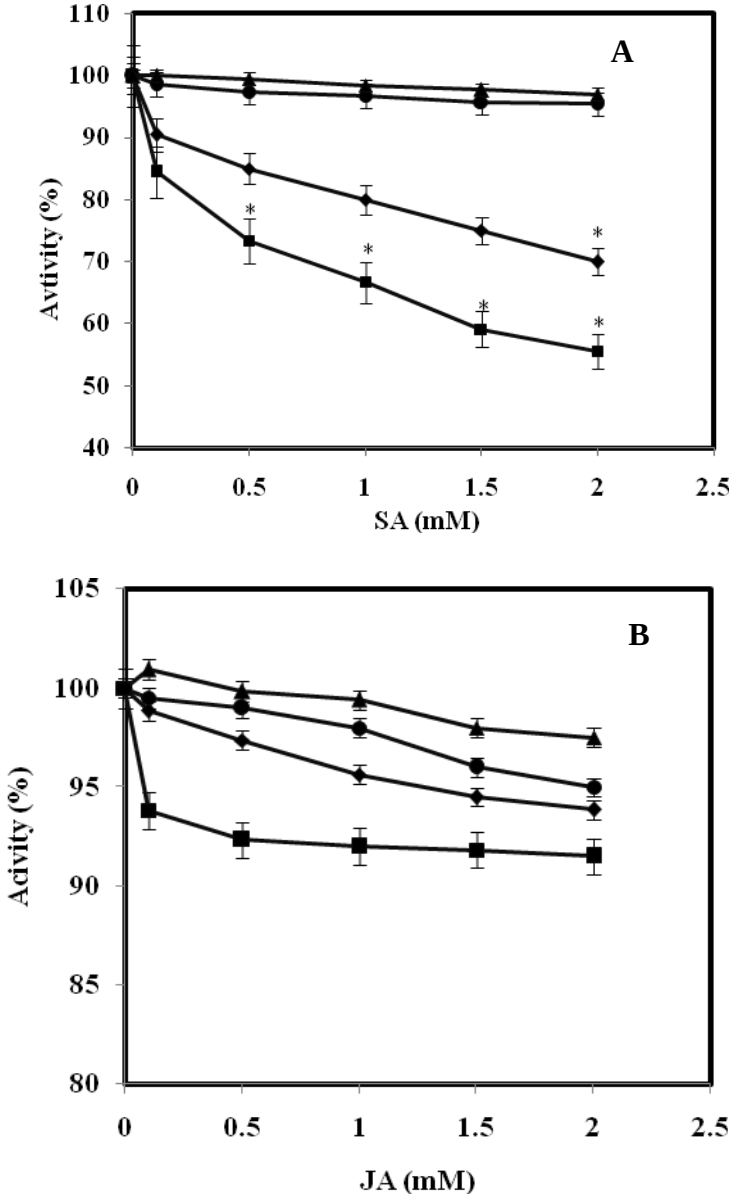


Fig. 3.2. Effect of SA (A) and JA (B) on NADH dehydrogenase (♦), succinate dehydrogenase (■), cytochrome oxidase (●) and F_0F_1 ATPase (▲) of isolated mitochondria. Enzyme activities were assessed as described in text. The data represents the mean ± S.D. (n = 3). (Significantly different from control at *$p < 0.05$).

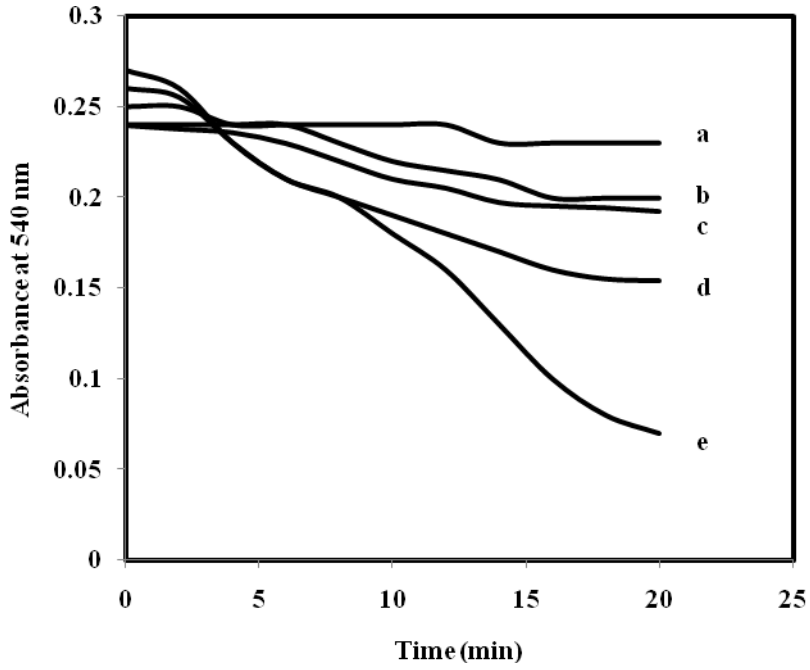


Fig. 3.3. Effect of SA and JA on the rate of swelling of mitochondria energized with succinate. Decrease in absorbance was measured at 540 nm in control mitochondria (a), in presence of 0.5 mM JA (b), in presence of 0.5 mM SA plus 50 µM cyclosporine A (c), in presence of Ca^{2+} (d), and in presence of 0.5 mM SA (e). The traces are the representative of three individual experiments.

into the supernatant and this efflux was inhibited by cyclosporine A. JA did not induce the release of cytochrome c when incubated with isolated mitochondria even at high concentrations (Fig. 3.4 A,B).

3.3.5. Effect of SA and JA on mitochondrial respiration and respiratory enzyme complexes *in vivo*

Mitochondria isolated from the SA-fed larvae exhibited normal state III respiration. However, state IV respiration was significantly increased (28.34 nmoles of O_2/min/mg protein) as compared with the control larvae (21.24 nmoles of O_2/min/mg protein), indicating the uncoupling behavior of the molecule under *in vivo* conditions (Table 3.3). RCI was also significantly affected in SA-fed larvae. Mitochondria prepared from the control and SA-fed larvae showed an RCI of 3.07 and 2.11 respectively (Table 3.3). None of the enzyme complexes were affected in SA-fed larvae, but a drop of about 50% activity was observed for F_0F_1 ATPase as compared with the control larvae. In contrast, mitochondria isolated from JA-fed larvae showed normal respiration, and no respiratory enzyme complex was affected (Table 3.3).

3.3.6. Lipid peroxidation, LDH leak, H_2O_2 content

Lipid peroxidation, LDH leak and H_2O_2 content were significantly higher in SA-fed larvae as compared with the control larvae. There was a proportional increase in these components in a time- and dose-dependent manner. The JA-fed larvae did not show any significant differences in these components as compared with the control larvae (Fig. 3.5 - 3.7).

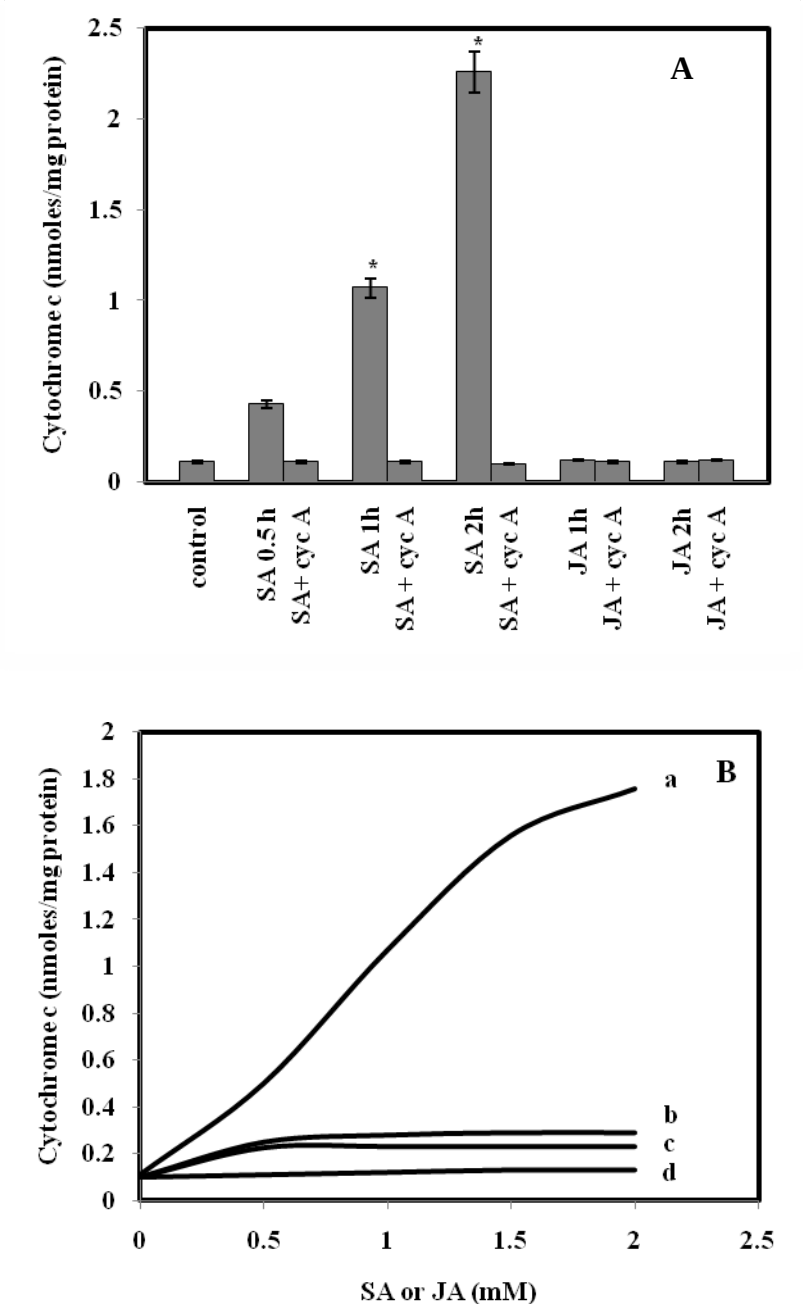


Fig. 3.4. (A) Time-dependent response for the effect of SA and JA on the release of cytochrome c (significantly different from control at *$p < 0.01$). (B) Dose-response of SA and JA on the release of cytochrome c. Intact mitochondria (about 1 mg protein) was incubated with 1mM of SA and JA in presence of 0.1 mM of Ca^{2+}, in presence or absence of cyclosporine A; and cytochrome c was estimated at different time intervals as described in the text. The data represents the mean ± S.D. (n = 3).

Table 3.3: *In vivo* effect of SA and JA on oxidation of succinate and respiratory enzyme complexes in mitochondria of *H. armigera*

	Control	**SA-fed larvae**	**JA-fed larvae**
State III respiration (nmoles/min/mg protein)	65.23 ± 2.88	59.89 ± 1.23	59.34 ± 0.236
State IV respiration (nmoles/min/mg protein)	21.24 ± 2.475	**28.34 ± 1.11***	20.67 ± 1.136
RCI	3.07 ± 1.163	**2.11 ± 1.108***	2.87 ± 0.207
Complex I (µmoles/min/mg protein)	59.75 ± 6.47	51.37 ± 0.68	56.28 ± 3.46
Complex II (µmoles/min/mg protein)	3.16 ± 0.41	2.83 ± 0.55	2.91 ± 0.51
Complex IV (µmoles/min/mg protein)	4.64 ± 0.69	4.56 ± 0.71	4.43 ± 0.95
Complex V (nmoles/min/mg protein)	5.73 ± 1.08	**3.04 ± 0.43***	6.11 ± 0.125

Values are mean ± SD (n = 3).

* Significantly differ from the control larvae at $p < 0.05$.

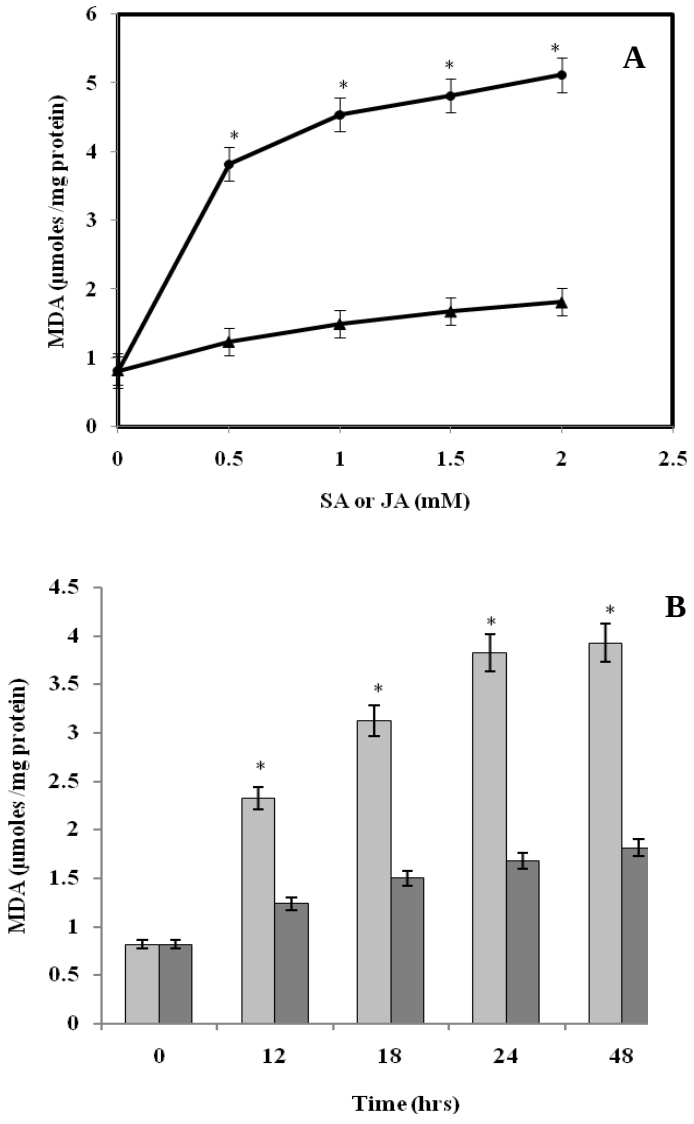


Fig. 3.5. (A) Dose-dependent response for lipid peroxidation in the larvae fed on artificial diet containing varying concentrations of SA (●) and JA (▲). (B) Time-dependent response for lipid peroxidation in the larvae fed on artificial diet containing 0.5 mM SA (▒), and 0.5 mM JA (■). The data represents the mean ± S.D. (n = 3). (Significantly different from control at *$p < 0.01$).

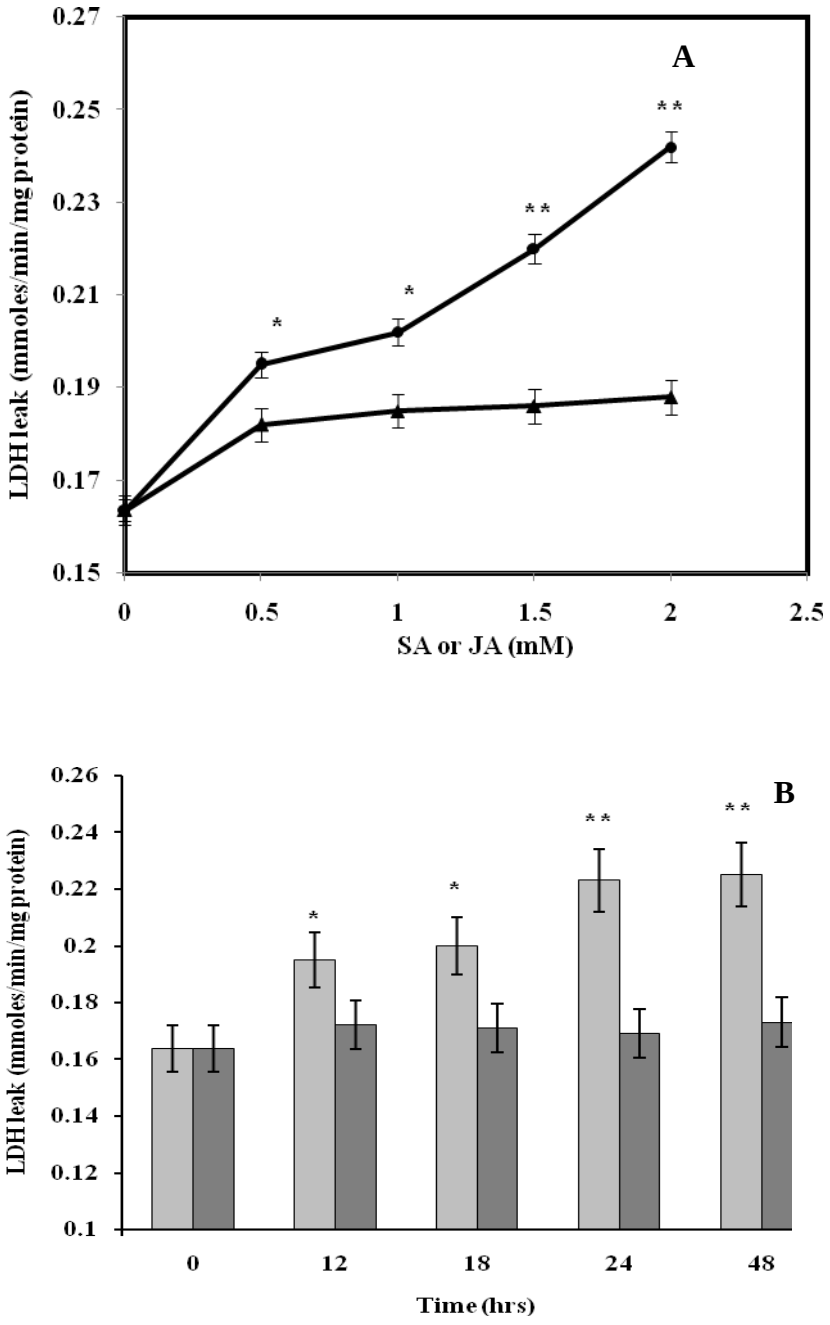


Fig. 3.6. (A) Dose-response for LDH leak in the larvae fed on artificial diet containing varying concentrations of SA (●) and JA (▲). (B) Time-dependent response for LDH leak in the larvae fed on artificial diet containing 0.5 mM SA (▒), and 0.5 mM JA (■). The data represents the mean ± S.D. (n = 3). (Significantly different from control at *$p < 0.05$ and **$p < 0.01$).

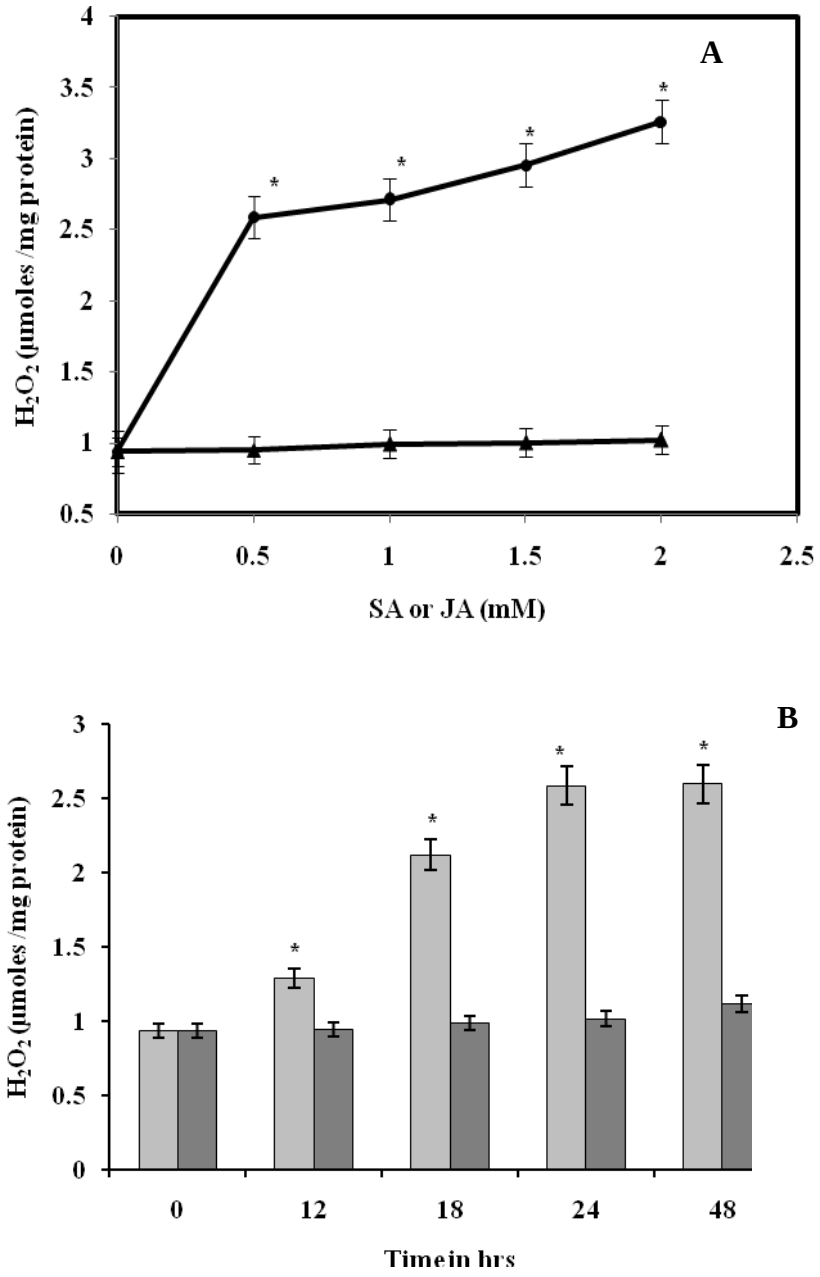


Fig. 3.7. (A) Dose-response for H_2O_2 production in the larvae fed on artificial diet containing varying concentrations of SA (●) and JA (▲). (B) Time-dependent response for H_2O_2 production in the larvae fed on artificial diet containing 0.5 mM SA (■), and 0.5 mM JA (■). The data represents the mean ± S.D. (n = 3). (Significantly different from control at *$p < 0.01$).

3.3.7. Bioassay of SA and JA

SA inhibited the larval growth in a dose-dependent manner, at 2 mM, the larval weight was decreased by 66%. However, there was no significant effect on the weights of larvae fed on JA amended diets (Fig. 3.8).

3.3.8. ATP Content

ATP content measured in SA- and JA-fed larvae indicated that SA inhibited the synthesis of ATP but JA did not (Fig. 3.9 – 3.13).

3.4. DISCUSSION

Plants react to pathogen attack by activating their defense mechanism in which plant cell signaling molecules play a key role in signal transduction pathways, thereby, resulting in induced resistance to herbivores. Salicylic acid (SA), Jasmonic acid (JA), nitric oxide (NO) and reactive oxygen species (ROS) (particularly H_2O_2) increase in abundance following pathogen recognition and each are important signaling molecules that promote and coordinate defense and hypersensitive response in plants (Cohen and Flescher, 2009). SA and JA are widely distributed in plants, and play an important role in systemic acquired resistance (Cohen and Flescher, 2009). These plant signal molecules raise the endogenous level of reactive oxygen species by regulating oxidative enzymes in plants (Xu and Tian, 2008). H_2O_2 and other reactive oxygen species, thus produced, serve as secondary messengers to induce the expression of plant defence related genes (Conarth et al., 1995). A number of reports have demonstrated the involvement of SA and JA in host plant resistance to herbivores (Metraux et al., 2002; Syeed et al., 2011). Topical application of SA and JA induces the production of pathogenesis related proteins, and subsequent resistance to phytopathogens (Bi et al., 1997). Exogenous application of SA (0.8 mM) caused the

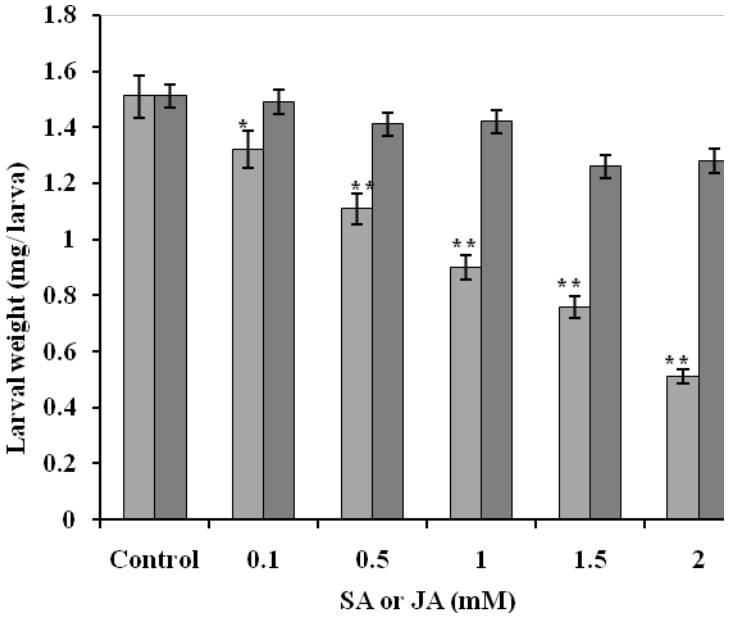
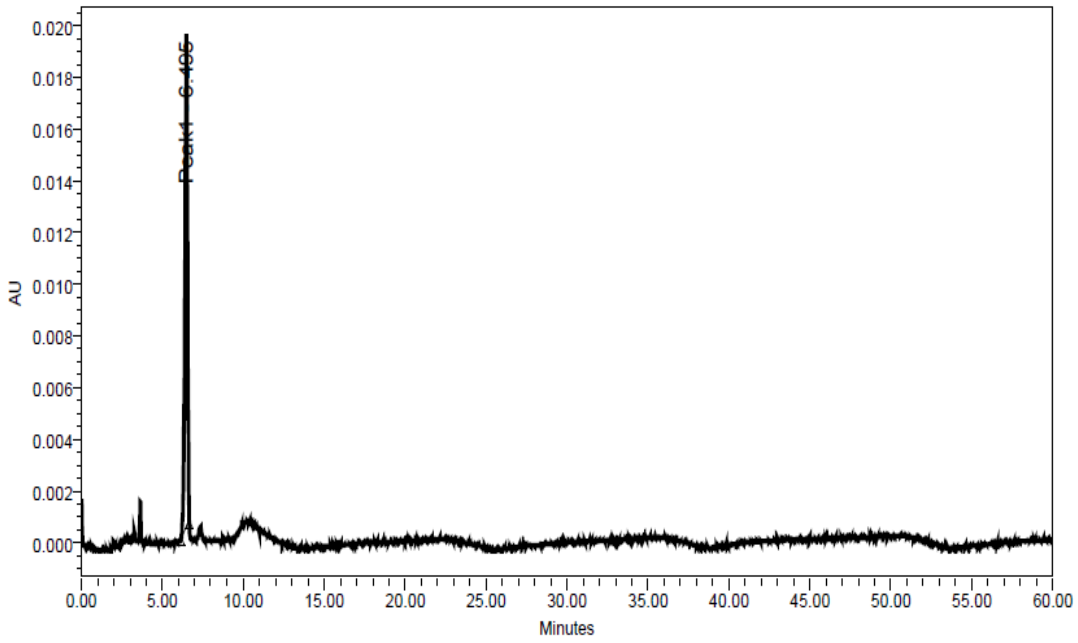
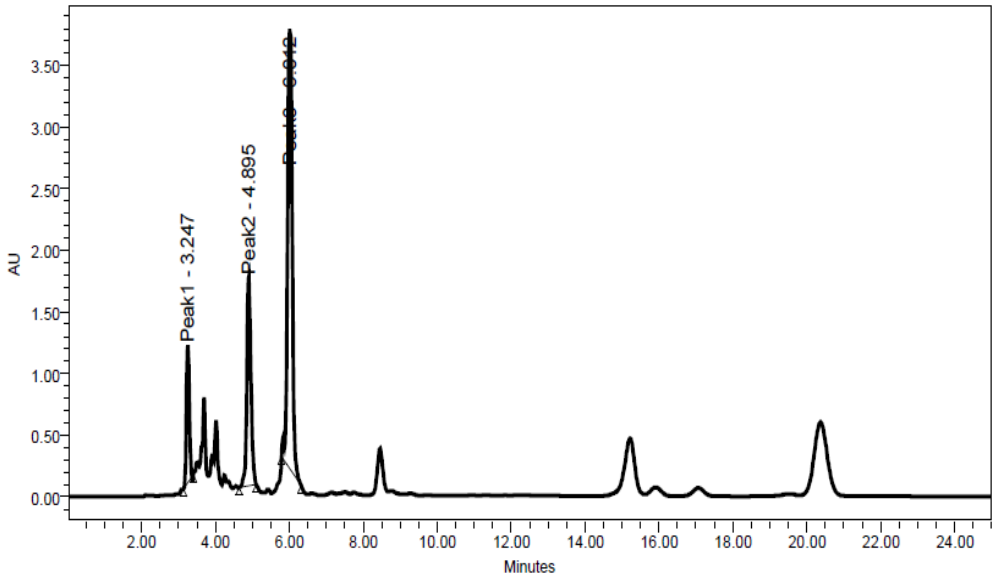


Fig. 3.8. Bioassays for SA and JA. Neonates were fed on artificial diet containing varying concentrations of SA (▫), and JA (▪). The data represents the mean ± S.D. (n = 3) (Significantly different from control at *p < 0.05, and **p < 0.01).



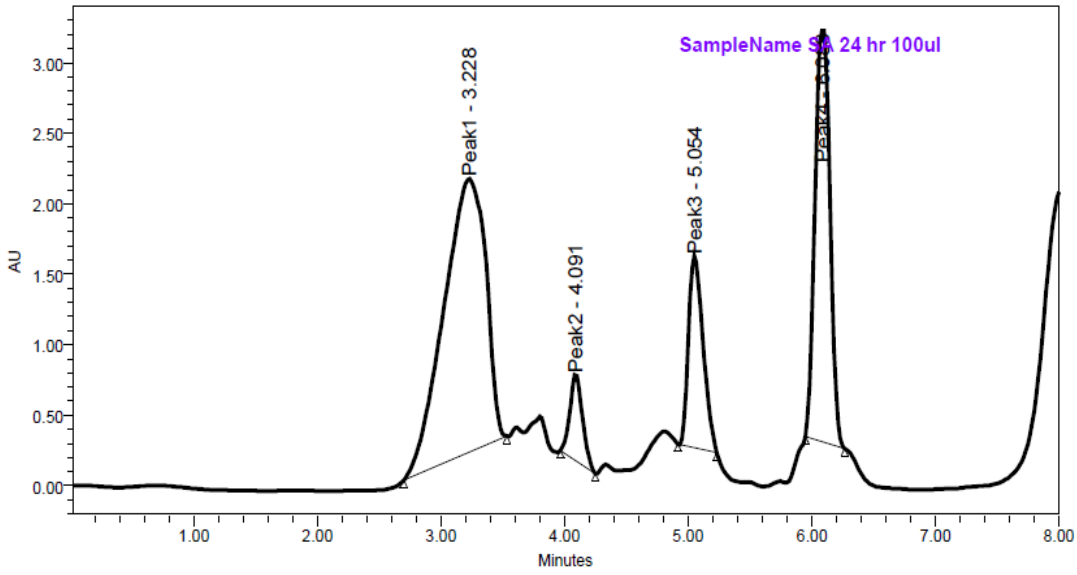
	Peak Name	RT	Area	% Area	Height
1	Peak1	6.495	203864	100.00	19075

Fig. 3.9. HPLC chromatogram for the standard ATP.



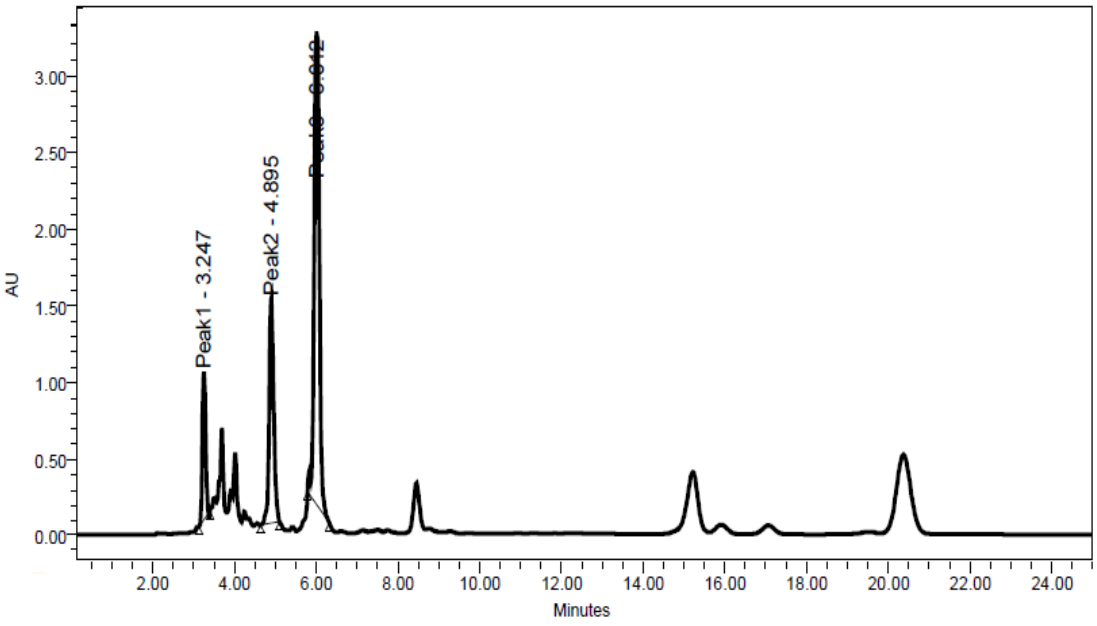
	Peak Name	RT	Area	% Area	Height
1	Peak1	3.247	6340586	12.42	1111368
2	Peak2	4.895	13100543	25.66	1699547
3	Peak3	6.012	31609052	61.92	3597421

Fig. 3.10. HPLC chromatogram for the ATP content in control larvae.



	Peak Name	RT	Area	% Area	Height
1	Peak1	3.228	45821078	53.26	1938087
2	Peak2	4.091	4315426	5.02	606709
3	Peak3	5.054	11214151	13.03	1360958
4	Peak4	6.088	24683124	28.69	2958713

Fig. 3.11. HPLC chromatogram for the ATP content in SA-fed larvae.



	Peak Name	RT	Area	% Area	Height
1	Peak1	3.247	6340586	12.42	1111368
2	Peak2	4.895	13100543	25.66	1699547
3	Peak3	6.012	31609052	61.92	3597421

Fig. 3.12. HPLC chromatogram for the ATP content in JA-fed larvae.

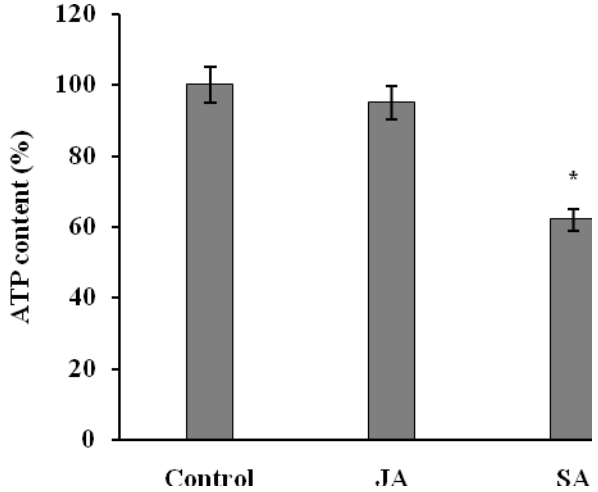


Fig. 3.13. Bar diagram representing ATP content in insects fed on diet congaing plant signaling molecule (Significantly different from control at *p < 0.05).

induction of protease inhibitors in chickpea plants which inhibited the proteases in *H. armigera* (Raju et al., 2009). The present studies demonstrated that SA also exhibit direct effects on insect growth through inhibition of respiration, but JA did not exhibit such effects on insect growth.

SA uncoupled the respiration of isolated mitochondria at lower concentrations (<1 mM), but inhibited at higher concentrations (>1 mM) (Fig. 3.1). SA also affected the RCI, which decreased with an increase in SA concentration, suggesting that it caused dysfunction of insect mitochondria *in vitro,* and impaired the mitochondrial respiration. Similar findings have earlier been reported for rat liver (Battaglia et al., 2005; Doi and Horie, 2010) and tobacco mitochondria (Norman et al., 2004). It was shown that SA disrupts mitochondrial function in a concentration dependent manner in tobacco suspension cells, at low concentrations, it acted as an uncoupler, whereas at higher concentrations it strongly inhibited electron flow (Norman et al., 2004). These effects were seen in both whole cells and isolated mitochondria. SA inhibited the activities of NADH dehydrogenase and succinate dehydrogenase in *H. armigera* (Fig. 3.2 A). Aspirin, a derivative of SA, has been reported as a uncoupler of oxidative phosphorylation in Yeast (Sapienza et al., 2008), and inhibitor of electron transport chain at complex I and complex II in rat liver mitochondria (Somsundaram et al., 1997). According to Battaglia et al., (2005), the most probable site for binding the SA is Fe^{3+} of Fe-S clusters of complex I. SA also inhibited F_0F_1 ATPase slightly (<5%) at 2 mM, which is not significant and these observations are similar to the results reported by Zhang and Ramirez (2000).

SA induced mitochondrial swelling in the presence of Ca^{2+} ions (Fig. 3.3), possibly due to the induction of membrane permeability transition (MPT), causing the accumulation of calcium ions in the matrix. SA-induced mitochondrial swelling was inhibited by cyclosporine A, a typical inhibitor of MPT, confirming the induction of MPT by SA. MPT is induced by the opening of a cyclosporine A sensitive permeability transition pore (PTP) in the mitochondrial inner membrane. The PTP resides at contact sites between the inner and outer membranes and its core components include the inner membrane-localized adenine nucleotide translocator (ANT), the outer membrane-localized voltage-dependent anion channel (VDAC) and the matrix-localized cyclophilin-D. Pore opening results in a loss of mitochondrial transmembrane potential, which is followed by an influx of water and solutes to the matrix. This causes matrix swelling and selective rupture of the outer mitochondrial membrane (because of its smaller surface area in comparison to the inner mitochondrial membrane), allowing the release of inter-membrane space proteins. Cyclosporin A (CsA) and bongkrekic acid (BA) are pharmacological inhibitors of PTP opening, acting by interaction with cyclophilin-D or ANT, respectively.

A key requirement for pore opening is the accumulation of Ca^{2+} in the mitochondrial matrix and susceptibility to Ca^{2+}-induced opening is influenced by numerous other aspects of mitochondrial status (Crompton, 1999). With this, the electrochemical gradient collapses and the corresponding increase in the electron flux along the respiratory chain results in an increase in oxygen uptake and consequently the production of reactive oxygen species, resulting in swelling and uncoupling. Enhancement of mitochondrial swelling by SA has been confirmed by spectrophotometrically and polarographically (Battaglia et al., 2005).

SA induced the release of cytochrome c in the presence of calcium ions, but cyclosporine A inhibited the same effect (Fig. 3.4 A,B). Release of cytochrome c is considered as an important parameter to measure apoptosis. Also, the pro- and antiapoptotic proteins may act by promoting or inhibiting PTP opening (Amirsadeghi, 2007). Presence of cytochrome c in the supernatant when isolated mitochondria incubated with SA thus confirms the SA-induced PTP opening. Release of cytochrome c by SA and aspirin has already been documented in rat liver mitochondria (Battaglia et al., 2005) and *Saccharomyces cerevisiae* cells (Sapienza et al., 2008), respectively. The impairment of mitochondrial functions observed with SA *in vitro* was mainly due to the induction of MPT and inhibition of electron transport chain through complex I and II. Determination of mitochondrial permeability transition by both cytochrome c release and mitochondrial swelling assays indicates that salicylic acid affects mitochondria isolated *H. armigera* directly, in a PTPC-mediated manner.

Mitochondria isolated from *H. armigera* larvae fed on diet containing SA exhibited uncoupling nature as evidenced by high state IV respiration, low RCI and a significant drop in the F_0F_1 ATPase activity (Table 3.3). The ATP content was also reduced in SA-fed larvae when compared with the control larvae, as evidenced by HPLC analysis (Fig. 3.13). Inhibition of both ATP synthesis and respiratory oxygen uptake in tobacco cell cultures within minutes of incubation with SA for whole cells and isolated mitochondria (Xie and Chen, 1999).

Mitochondrial electron transport is associated with the generation of ROS such as superoxide and H_2O_2. Because ROS can damage macromolecules, and their cellular levels

are managed through avoidance and scavenging mechanisms (Mittler et al., 2004). In animals, complexes I and III likely represent the primary sites of ROS generation (Møller, 2001). The relative importance of these two sites of ROS generation and the factors influencing their rates of ROS production are largely unknown but an important generalization is that ROS formation increases as the ETC becomes more highly reduced. Lipid peroxidation, LDH leak and H_2O_2 content were measured as the markers for oxidative stress. Significant increase in lipid peroxidation, LDH leak and the H_2O_2 content were observed in SA-fed larvae (Fig. 3.5 – 3.7). SA has been reported to generate H_2O_2 and other reactive oxygen species in isolated rat liver mitochondria (Battaglia et al., 2005) that resulted in induction of lipid peroxidation in cell membranes and LDH leak from mitochondria (Doi and Horie, 2010). These results are similar to SA-induced oxidative stress observed in isolated rat liver hepatocytes (Doi and Horie, 2010).

Thus, the SA-induced lipid peroxidation accompanied by high levels of LDH activity and H_2O_2 content in SA-fed larvae supports the assumption that SA induces oxidative stress in cells. A dose-response relationship was evident in case of SA on the oxidative stress. Bioassays indicated that SA affected the larval growth (Fig. 3.8), which can be explained in terms of increased oxidative stress, and impairment in the mitochondrial function as evidenced by the uncoupling effect on mitochondrial respiration, decrease in the respiratory control index (RCI), inhibition of F_0F_1 ATPase activity under *in vivo* conditions. Since the mitochondrial respiratory chain produces the majority of ATP content of the cells, an impairment in the mitochondrial respiration could adversely affect the energetic state of the cell.

JA showed no effect on mitochondrial respiration. *In vitro* and *in vivo* studies showed that JA has no effect on the respiratory parameters (State III, IV respiration, RCI and P/O ratios) of mitochondria of *H. armigera* with any of the used substrates (Table 3.2, 3.3). ROS, including hydrogen peroxide, hydroxyl radicals, and superoxide anions, are produced in cells as a consequence of electron leakage from the electron transport chain and can have detrimental effects on cells, causing lipid peroxidation, DNA adduct formation, and protein oxidation (Goetz and Luch, 2008). ROS levels are significantly elevated in cancer cells when compared with their nontransformed counterparts (Trachootham et al., 2006). Oh and colleagues (2005) showed that treatment of glioma cells with jasmonates leads to elevated ROS levels.

We therefore, investigated whether jasmonic acid could induce the production of oxidative stress markers in terms of H_2O_2 content, lipid peroxidation and LDH leak. Interestingly we could not find a relationship between JA-fed larvae and oxidative stress, more than 24 hour-exposure of JA even at higher concentration of 2 mM did not induced the production of oxidative stress markers *in vivo*, thus JA had a contrary effect in insect cells when compare to SA-fed larvae (Fig. 3.5 – 3.7). Jasmonates are known to induce mitochondrial membrane depolarization, osmotic swelling, and the release of cytochrome c in cancerous cells. Determination of mitochondrial permeability transition by both mitochondrial swelling (Fig. 3.3) and cytochrome c release (Fig. 3.4) assays indicates that JA did not affected mitochondria isolated from JA-fed larvae. Bioassays also indicated that JA did not affect the larval growth (Fig. 3.8).

It is known that JA specifically causes mitochondrial dysfunction in cancerous cells by disrupting membrane potential, enhancing swelling by inducing MPT, cytochrome c

release, and induces the apoptotic pathway leading to cell death (Rotem et al., 2005). Increasingly, natural products from plants are being identified as possessing antitumor activities. Salicylic acid and its synthetic derivative-acetyl salicylic acid, i.e., aspirin, have been studied as potential anti-cancer agents. Salicylate suppressed the proliferation of various types of cancer cells, including lymphoblastic leukemia, prostate, breast and melanoma human cancer cells (Fingrut and Flescher, 2002; Sotiriou et al., 1999). Wide spectrum of malignancies, including three of the most important human cancers-prostate, breast and lung (Fingrut and Flescher, 2002; Kim et al., 2004), exhibited sensitivity to the cytotoxic effects of methyl-jasmonate.

Although both, SA and JA share the common function of anticancer activity by inducing the mitochondrial dysfunctions and oxidative stress, SA affected the insect mitochondria but JA did not. JA is specific to cancer cell mitochondria, and differentiates between normal cells and cancer cell mitochondria. Since the composition and function of mitochondria of cancer cells differ from normal cells in having certain characteristics such as high membrane potential, possibly a module expression of the MPT component and enhanced rates of ATP generation through glycolysis rather than through oxidative phosphorylation (the Warburg effect) (Rotem et al., 2005). Hence, JA did not exhibit any effect on mitochondria of *H. armigera* and did not induce any oxidative stress in JA-fed larvae. Similar observations have earlier been reported by Rotem et al., (2005) that, JA induces MPT in cancerous cells but not in normal cells.

In conclusion, SA induced mitochondrial dysfunction and oxidative stress in *H. armigera* under *in vitro* and *in vivo* conditions, suggests that SA affect the growth of Lepidopteran insects. Further, SA when applied exogenously could act as antifeedant and

affect the growth and development of larvae by inducing mitochondrial dysfunction and oxidative stress in *H. armigera*. This capacity to induce deleterious effects on insect growth is expected to work in concert with its proven induced-resistance in plants, making SA an excellent candidate for the control of pests. Although JA did not affected the larval growth, but is known to induce resistance in plants against insects. Thus there is a need to develop strategies to exploit SA and JA for the control of Lepidopteron pests, and to reduce our dependence on chemical pesticides for crop production.

Chapter IV

Characterization of dihydrolipoamide dehydrogenase from the mitochondria of Helicoverpa armigera

4.1. INTRODUCTION

The mitochondrial enzyme, dihydrolipoamide dehydrogenase (E.C.1.8.1.4) belongs to the group of flavin containing pyridine nucleotide disulfide oxidoreductases (Huo et al., 2010). DHLDH is a component of multienzyme complexes such as pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KGDH) and branched chain ketoacid dehydrogenase (BCKADH). These complexes catalyze the conversion of α-ketoglutarate to sucinyl CoA and pyruvate to acetyl-CoA, which is subsequently used in fatty acid biosynthesis or is converted into citrate for the oxidation in TCA cycle. All DHLDHs are stable homodimers, each monomer possessing a noncovalently but tightly bound flavin adenine dinucleotide (FAD), a transiently bound NADH or NAD^+ molecule, and two redox active cysteine residues (Brautigam et al., 2005). The enzyme functions physiologically to catalyze the NAD^+-dependent oxidation of the dihydrolipoyl cofactor covalently linked to the acetyl transferase components.

The enzyme DHLDH has been purified from a number of prokaryotes, eukaryotes, and some archaebacteria. However, DHLDH has been characterized only from limited number of insect species. Since insects represent the most abundant and diverse group of organisms in any Phylum, their number and diversity far exceeds the species in all other Phyla combined (Novotny et al., 2002), they are interesting candidates to study evolutionary, genetic and biochemical properties of multienzyme complexes. In the present study, we report the characterization of purified DHLDH from the mitochondria of *H. armigera,* its N-terminal sequence analysis and inhibition of enzyme activity by arsenical compounds. Information on the structure and function of DHLDH will be important for developing strategies to overcome insecticidal resistance in *H. armigera* populations in different agro-ecosystems.

4.2. MATERIALS AND METHODS

4.2.1. Glassware cleaning

Glassware was first cleaned in tap water and soaked in chromic acid solution (15 % potassium dichromate in 25 % concentrated sulfuric acid) for 12 h. This was followed by thorough tap water washing in detergent solution. The glassware was then dried in hot air oven after successive washings with deionized and distilled water.

4.2.2. Chemicals

α-Lipoamide, CM-Sephadex, CHAPS, hydroxylapatite, NADH, sodium arsenite and melarsoprol were purchased from Sigma Aldrich. Tris-base, pyruvate and 5,5¹-dithio-bis(2-nitro benzoic acid) were purchased from Himedia, India, while the other chemicals were of analytical grade.

4.2.3. Insect culture

Insecticide resistant population of *H. armigera* was supplied by Dr. S.S. Udikeri, Agriculture Research Station, UAS, Dharwad, Karnataka, India. The larvae were maintained in laboratory on artificial diet as described in earlier. The larvae were washed in chilled distill water, their gut contents removed, and body membranes taken for isolation of DHLDH.

4.2.4. Isolation of enzyme

For the isolation of DHLDH, the mitochondria were isolated from body membranes of fourth- and fifth-instar larvae of *H. armigera* according to the modified method of Chamberlin (2004). This entailed gently homogenizing the body membranes of the larvae in extraction medium of 50 mM Tris HCl buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA. The homogenate was centrifuged at 800 g for 5 min, and the supernatant was

centrifuged at 12,000 g for 10 min. The pellet containing mitochondria was suspended in 50 mM Tris HCl buffer, pH 7.4, containing 1 mM EDTA and 1% CHAPS, and kept for stirring at 4 ºC overnight. The extract was centrifuged at 15,000 g for 30 min, and the supernatant was used as source of DHLDH.

4.2.5. Enzyme assays

The DHLDH activity was assayed spectrophotometrically in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1 mM dithioerythritol, 0.025 mM NADH and 0.5 mM lipoamide, in a total reaction volume of 1 ml in presence of purified protein at 30 ºC (Huo et al., 2010). The reaction was started by addition of lipoamide, and NADH oxidation was measured as change in absorbance at 340 nm over time. The PDH complex activity was measured spectrophotometrically at 30 ºC according to Heine and Steinbuchel (1994). Reduction of NAD was measured at 340 nm and the reaction started with 1.5 mM pyruvate. One unit of enzyme activity was expressed as µmoles of NADH consumed or produced per minute at 30 ºC. Protein concentration was determined by Lowry's method (1951).

4.2.6. Purification of DHLDH

To purify DHLDH, ammonium sulfate was added to 50 ml of crude extract (1,100 mg of protein) to give 40% saturation. The solution was kept for overnight stirring at 4 ºC, and the precipitate was removed by centrifugation for 30 min at 10,000 g, and the supernatant was brought to 60% ammonium sulfate saturation. After overnight stirring at 4 ºC, salt precipitated proteins were collected by centrifugation for 30 min at 10,000 g, and dissolved in buffer A (50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1 mM dithioerythritol), and dialyzed for 18 h against the preceding buffer at 4 ºC. This pool was applied to hydroxylapatite column (2 x 10 cm) previously equilibrated with buffer A. After

the column was washed with 100 ml of the same buffer, proteins were eluted with a step gradient of 0 to 1.0 M potassium phosphate buffer, pH 7.4, and the fractions were collected each of 3 ml. DHLDH activity was eluted at 0.4 M and 0.5 M phosphate buffer fractions, and the active fractions were pooled, concentrated, and dialyzed against buffer B (50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM dithioerythritol), and applied to a column of CM-Sephadex (2 x 10 cm) previously equilibrated with the preceding buffer. After the column was washed with 40 ml of buffer B, proteins were eluted from the column with a step gradient of 0 to 1 M NaCl, and fractions of 2 ml each were collected. DHLDH activity was eluted at 0.5 M and 0.6 M NaCl. Fractions containing DHLDH activity were pooled, concentrated using Viva cell 250 (MW 50,000) to a final volume of 2.5 ml, and dialyzed against buffer B. DHLDH in the purified preparation was tested for homogeneity by SDS-PAGE (Laemmli, 1970).

4.2.7. Electrophoresis

The molecular weight of the protein under denaturing conditions was determined by 10% SDS-PAGE (Laemmli, 1970). Native molecular weight of the protein was determined by native polyacrylamide gel electrophoresis, in polyacrylamide gel gradient formed from 5 to 15% using Sigma molecular weight markers in the range of 29 to 205 kDa (Margolis and Kenrick, 1967). DHLDH activity was detected by incubating the gel in a reaction mixture containing 2 mM lipoamide, 0.4 mM NADH, 0.5 mM 5,5¹-dithio-bis(2-nitro benzoic acid) and 1 mM EDTA in 50 mM potassium phosphate buffer, pH 7.4, at 37 ºC, until yellow bands appeared (Dietrichs and Andreesen, 1990).

4.2.8. Kinetic studies and substrate specificity

Kinetic studies were carried out in 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM dithioerythritol in a total reaction volume of 1 ml at 30 ºC. NADH concentration varied from 0.05 mM to 0.4 mM at 2.5 mM lipoamide, for determination of K_m of NADH. And lipoamide concentration varied from 0.5 mM to 4 mM at 0.25 mM NADH, for determination of K_m of lipoamide. Substrate specificity was tested using both the reduced pyridine nucleotides, NADH and NADPH for the reduction of lipoamide. To determine the mechanism of enzyme catalyzed reaction, double-reciprocal plots were drawn in the direction of oxidation of NADH with varying NADH concentrations at fixed lipoamide concentration of 1 mM, 1.5 mM and 2 mM.

4.2.9. Influence of pH, temperature and metal ions

Influence of pH on the activity of DHLDH was studied in the above mentioned reaction mixture at different pH values ranging from 4 to 10. The enzyme was incubated at different temperatures ranging from 20 - 80 ºC for 15 min, to determine the effect of temperature on enzyme activity. Metal ions such as Na, Mg, K, Ca, Mn, Fe, Cd, Ag, Zn, Co, Pb, Cu, Hg, arsenite and melarsoprol at 1 mM concentration were incubated with the enzyme for 15 min at room temperature to determine the effect of different metal ions on the catalytic activity of the enzyme.

4.2.10. Identification of coenzyme

The purified enzyme was denatured at 100 ºC in dark to liberate the bound flavin, and centrifuged at 15,000 g for 15 min. The released flavin was analyzed by thin layer chromatography (Dietrichs and Andreesen, 1990). Protein spectra were obtained in Schimadzu spectrophotometer keeping 50 mM phosphate buffer, pH 7.4, as reference.

4.2.11. N-Terminal sequencing

For determination of N-terminal sequence, purified DHLDH protein was subjected to SDS-PAGE, then the protein was transferred to polyvinylidenedifluoride membrane, and the membrane was stained with Coomassie blue R250. The enzyme was detected in gel by activity staining (Dietrichs and Andreesen, 1990), areas of DHLDH from the membrane were excised and sent for sequence analysis using Edman degradation based Procise protein sequencing system at HSC Advanced Protein Technology Center, Department of Structural Biology and Biochemistry, Toronto, Canada.

4.3. RESULTS

4.3.1. Purification of DHLDH

The cells were disrupted manually to collect mitochondria and treated with CHAPS buffer to release any membrane bound DHLDH. The enzyme extract was fractionated by ammonium sulphate precipitation, and the salt precipitated proteins were subjected to hydroxylapetite (Fig. 4.1) and CM-Sephadex (Fig. 4.2) after dialysis. The DHLDH was eluted as a single peak from CM-Sephadex, and gave a single band when analyzed by SDS-PAGE. From 1,100 mg of total protein, 4 mg of enzyme was obtained with 17.98-fold-increased specific activity and 10.53% recovery. Specific activity of purified enzyme was 18.7 U/mg of protein (Table 4.1).

4.3.2. Molecular weight of DHLDH

Subunit molecular weight of DHLDH was estimated to be 66 kDa. The native molecular weight of the protein was determined to be 128 kDa, suggesting that the protein existed as a dimer (Fig. 4.3).

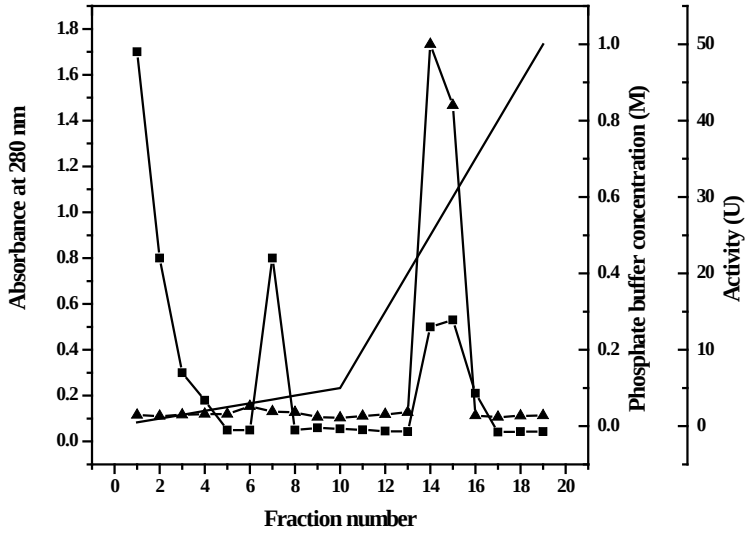


Fig. 4.1. Elution profile for the protein and DHLDH activity on hydroxylapatite column. Protein (■), enzyme activity (▲), phosphate buffer concentration (—).

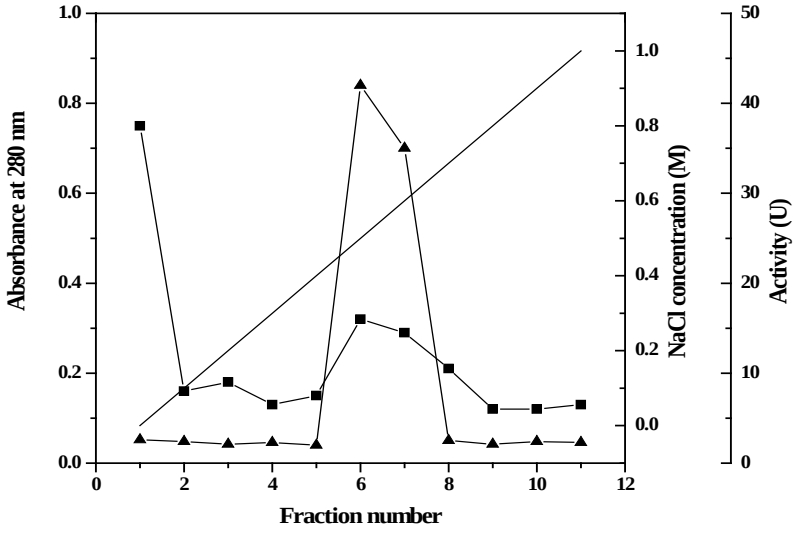


Fig. 4.2. Elution profile for the protein and DHLDH activity on CM-Sephadex column. Protein (■), enzyme activity (▲), NaCl concentration (—).

Table 4.1: Purification of DHLDH from mitochondria of *H. armigera*

	Volume (ml)	Total protein (mg)*	Total activity (U)*	Specific activity (U/mg)*	Purification fold	Yield (%)
Crude extract	50.0	1100 ± 5.25	1146.30 ± 10.12	1.04 ± 0.02	1.00	100.00
NH_4SO_4 precipitation	16.0	192 ± 2.89	867.84 ± 5.78	4.52 ± 0.43	4.34	75.70
Hydroxylapatite	6.0	48 ± 1.54	404.16 ± 2.87	8.42 ± 0.77	8.09	35.25
CM-Sephadex	2.5	4 ± 0.19	120.80 ± 1.99	18.70 ± 1.22	17.98	10.53

*Values in the table represents Mean ± SE (n=3).

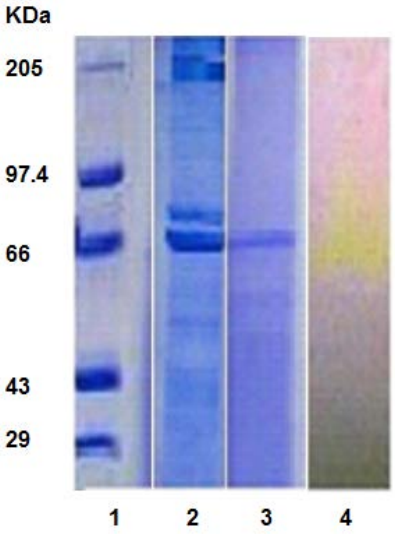


Fig. 4.3. SDS-PAGE pattern of DHLDH of *H. armigera*. Lane 1, molecular weight markers (29 to 205 kDa); lane 2, CHAPS solublized mitochondrial pellet; lane 3, purified enzyme (CM-Sephadex fraction); lane 4, gel stained for the activity of the enzyme (staining procedure mentioned in text).

4.3.3. Kinetic studies and substrate specificity

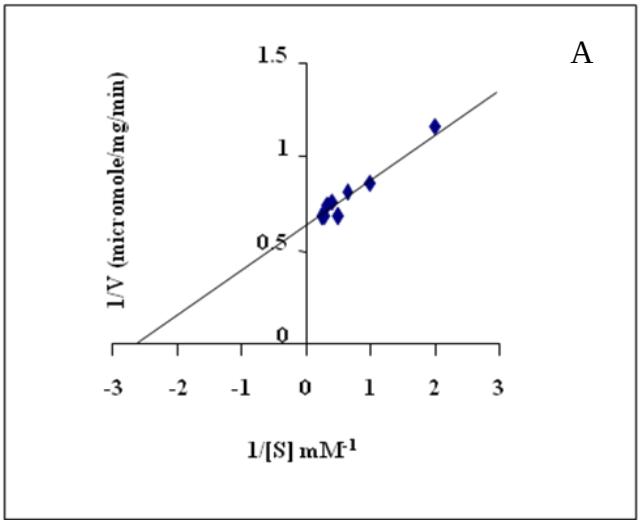
The velocity of NADH dependent reduction of lipoamide showed a hyperbolic dependence on the NADH and lipoamide concentration. Estimated Michaelis-Menten constants (K_m) were 0.083 mM for NADH and 0.4 mM for lipoamide, maximum velocity (V_{max}) of 61.25 U was observed in both the cases, i.e., with varying concentration of lipoamide and NADH (Fig. 4.4). Substrate specificity tested in the direction of oxidation of reduced pyridine nucleotides indicated that the enzyme was highly specific to NADH as a coenzyme, and did not use NADPH. Double reciprocal plots drawn for the enzyme activity yielded a series of parallel lines, which fitted ping-pong mechanism of catalysis (Fig. 4.5).

4.3.4. Influence of pH, temperature and metal ions

Acetate buffer, Tris-HCl and bicarbonate buffer were used for acidic, neutral and basic pH of the reaction mixture, respectively. Enzyme activity showed parabolic dependence on pH, with an optimum pH of 7.4 (Fig. 4.6). The *H. armigera* DHLDH is quite resistant to thermal inactivation, the enzyme activity was unaffected even at high temperature of 80 ºC. Metal ions such as Cu, Cd, Zn, Co, Pb, Ni, Hg, arsenite and melarsoprol completely inhibited the enzyme activity, whereas Ca and K enhanced the enzyme activity up to 50% (Table 4.2).

4.3.5. Identification of coenzyme

The spectra of DHLDH showed absorption maxima at 420 nm, 455 nm and 475 nm (Fig. 4.7). The absorbance at 455 nm decreased when the enzyme was reduced with 2 mM NADH. Addition of sodium dithionite resulted in complete decrease of absorbance at 455 nm. These types of spectra are typical of flavoproteins (Lohrer and Krauth-Siegel, 1990).



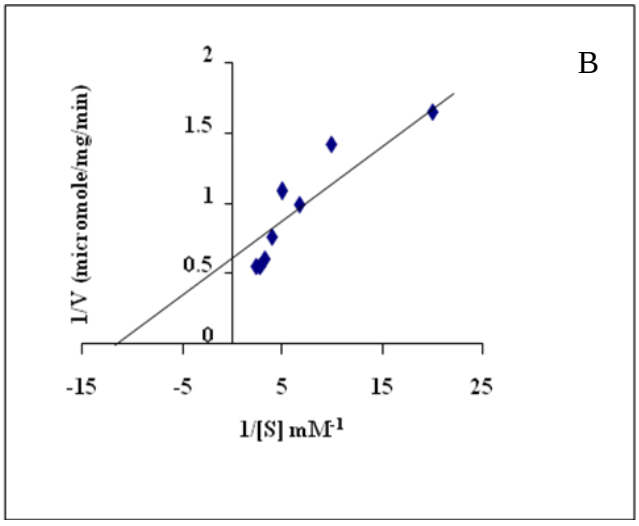


Fig. 4.4. Double reciprocal plots for the initial velocity of the enzyme with varying concentrations of lipoamide at fixed concentrations of NADH (A), and with varying concentrations NADH of at fixed concentrations of lipoamide (B).

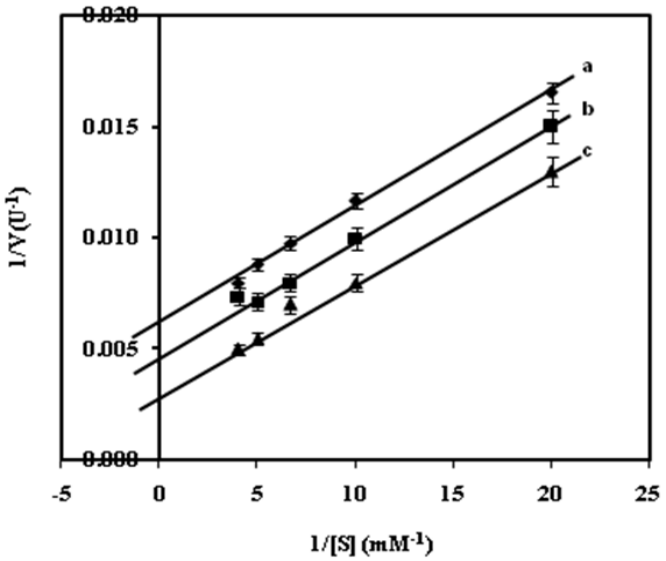


Fig. 4.5. Double reciprocal plots for the initial velocity of the enzyme with varying concentration of NADH at fixed concentrations of lipoamide, 1 mM (a), 1.5 mM (b) and 2 mM (c), in 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 1mM dithioerythritol.

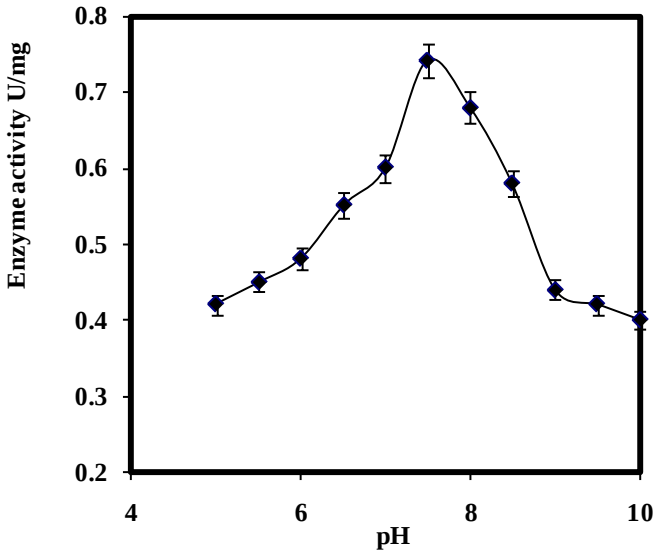


Fig. 4.6. Effect of pH on the activity of DHLDH of *H. armigera.* The enzyme activity was found to be Enzyme activity showed maximal activity at pH 7.4.

Table 4.2: Effect of metal ions on dihydrolipoamide dehydrogenase activity of *H. armigera*

Metal (1 mM)	Activity (%)*
Control	100.00
$CaCl_2$	156.67
KCl	153.34
NaCl	113.33
$MgCl_2$	106.67
$MnCl_2$	106.67
$FeSO_4$	90.00
$AgNO_3$	33.33
$FeCl_3$	20.00
$CuSo_4$	3.76
$PbSO_4$	3.79
$ZnSo_4$	3.72
$CdCl_2$	3.72
$HgCl_2$	4.09
$CoCl_2$	3.76
Sodium arsenite	3.79
Melarsoprol	3.45

*The values are means of three different assays.

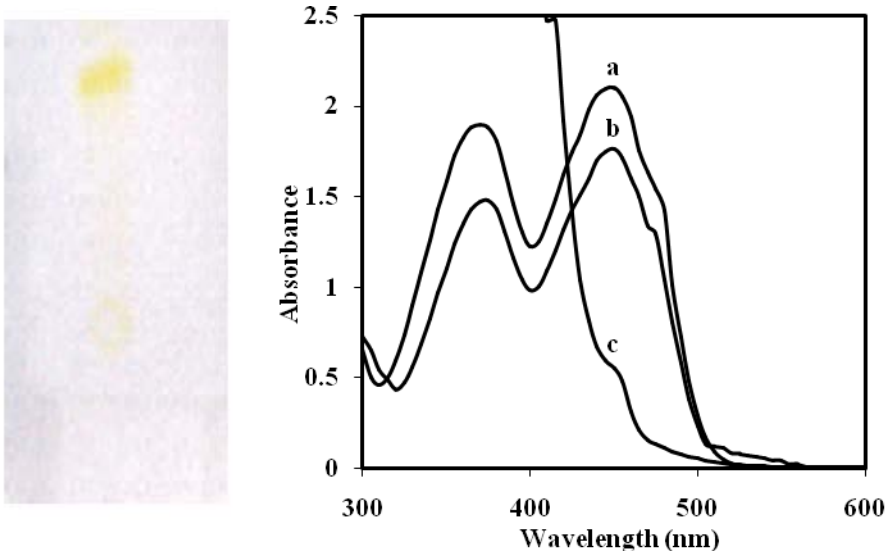


Fig. 4.7. (A) Isolation of coenzyme by thin layer chromatography. (B) Absorption spectra of DHLDH of *H. armigera*. The oxidized enzyme in 50 mM phosphate buffer, pH 7.4 (a) was reduced with 2 mM NADH (b) and excess sodium dithionite (c).

4.3.6. N-Terminal sequence of DHLDH

The N-terminal sequence was blasted with mammal and arthropod DHLDH sequences as shown in Figure 4.8, using NCBI website (www.ncbi.nlm.nih.gov). Sixteen amino acids were sequenced, and about 10 positions were found to be identical with the sequence of mammalian and arthropod DHLDH. The sequence of DHLDH of *H. armigera* showed homology with the oxidoreductase region of mammal's DHLDH (Otulakowski and Robinson, 1987) and the stretch of sequence GAGPGG represented the binding site for pyrophosphate group of FAD (Lohrer and Krauth-Siegel, 1990).

4.4. DISCUSSION

The present study describes the isolation of DHLDH from the mitochondria of *H. armigera*. It is a flavoprotein with a broad specificity for electron acceptors. In addition to the activity associated with lipoamide, it also acts as a diaphorase, transferring electrons from NADH to oxygen. It is also capable of using artificial electron acceptors such as methylene blue, dichlorophenol indophenol, ferricyanide, quinines and cytochrome c (Williams, 1976; Tsai et al., 1983). DHLDH also catalyzes transhydrogenase reactions with pyridine nucleotides (Tsai et al., 1983) and reduces intracellular thiol groups. It has also been reported that, in mitochondria, dihydrolipoic acid play an important role in maintaining the ATP content in the cell i.e., they enhance the ATP synthase activity and prevent ATPase activation (Zimmer et al., 1991), and also maintain the membrane proteins in proper redox state (Serrano, 1992). Although the enzyme isolated from different sources exhibits similar catalytic mechanisms, there are some distinctive, quantative differences between the bacterial and insect lipoamide dehydrogenase with few resemblances with mammalian DHLDH.

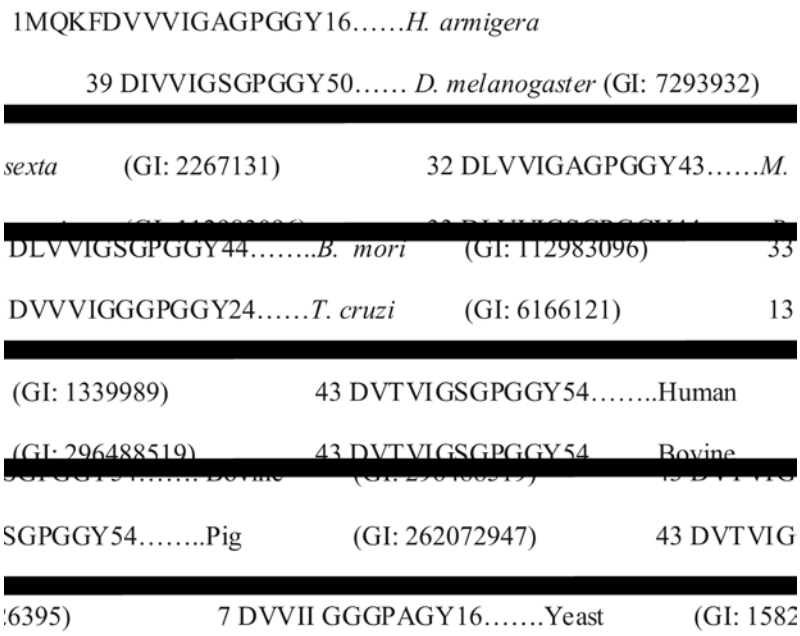


Fig. 4.8. Alignment of the N-terminal sequence of *H. armigera* DHLDH with DHLDHs of *Drosophila melanogaster*, *Manduca sexta*, *Bombyx mori*, *Trypanosoma cruzi*, human, bovine, pig, yeast, and *Mycoplasma mycoides.*

DHLDH from *H. armigera* was found to be specific to NADH and showed no activity with NADPH. Based on substrate specificity, Dietrichs and Andreesen (1990) classified bacterial DHLDH into four types, one utilizing NADH alone, second utilizing both NADH and NADPH, third utilizing NADPH alone and the fourth utilizing both NADH and NADPH with preference for NADPH. The DHLDH isolated from *H. armigera* thus represents the classical type of enzyme utilizing only NADH as its substrate, having a M_r of 66 kDa per subunit (Fig. 4.3). The native molecular weight of the protein was found to be 128 kDa, suggesting that the protein existed as homodimer in association with ketoacid dehydrogenase complexes. The molecular weight of the enzyme reported from *Trypanosoma cruzi* (Lohrer and Krauth-Siegel, 1990), *Manduca sexta* (Pullikuth and Gill, 1997) and human liver (Ide et al., 1967), have a subunit M_r of 55 kDa, 51 kDa, and 138 kDa (native molecular weight), respectively, which are around the M_r of DHLDH of *H. armigera*. The enzyme also reduced 2,6-dichloroindophenol and potassium ferricyanide using NADH as physiological electron donor. DHLDH is known for its broader specificity for artificial electron acceptors (Youn et al., 1998).

The enzyme showed a parabolic dependence on pH of the medium, with an optimum pH of 7.4 (Fig. 4.6). In alkaline conditions, the enzyme activity gradually decreased due to denaturation of the enzyme. Optimum pH for DHLDH from *E. coli* is 8, *T. cruzi* 6.75, and human liver 6.5, which is around the neutral pH. The enzyme was resistant to thermal inactivation, the enzyme activity was not affected even at high temperature up to 80 ˚C. According to Schmincke-ott and Bisswanger (1981), the transition state activation energy is 43 kJ/mol for the complex bound form and 74.8 kJ/mol for the individual E3 component in

E. coli K12 strain. This reflects the stability of the component and its ability to withstand such high temperatures to overcome the transition state barrier.

Michaelis-Menten constants (K_m) for the substrates lipoamide and NADH were 0.4 mM and 0.083 mM, respectively (Fig. 4.4). These values are similar for the substrates from other organisms, e.g., K_m for NADH with human liver (Ide et al., 1967) and *T. cruzi* (Lohrer and Krauth-Siegel, 1990) DHLDH is 0.023 mM and 1.33 mM, respectively, while a high K_m of 5 mM has been reported for lipoamide with DHLDH of *T. cruzi* (Lohrer and Krauth-Siegel, 1990) and bovine liver mitochondria (Lusty, 1963). A series of parallel lines were obtained when a double reciprocal plot was drawn (Fig. 4.5). Such behavior is exhibited by enzymes operated by ping pong mechanism, as proposed in some bacteria (Williams, 1976) and in eukaryotes (Massey et al., 1960; Reed, 1973). This also implied that the binding sites for NADH and disulfide substrate are separated on the enzyme by isoalloxazine ring of FAD (Thieme, 1981). The spectral properties found with DHLDH of *H. armigera* (Fig. 4.7) are in accordance with those of DHLDH of *T. cruzi* (Lohrer and Krauth-Siegel, 1990) and pig (Massey et al., 1960).

The N-terminal sequence of Ha-DHLDH was identical to bacterial, arthropod and mammalian DHLDH (Fig. 4.8). The sequence lies in FAD binding domain of the enzyme, which is highly conserved, as observed in the E3 component of *M. sexta* (Pullikuth and Gill, 1997) and *T. cruzi* (Lohrer and Krauth-Siegel, 1990). The enzyme also showed homology to the conserved sequence of human erythrocyte glutathione reductase (Thieme, 1981) and *Pseudomonas aeruginosa* mercury (II) reductase (Brown et al., 1983). Evidences has shown that DHLDH present in cytoplasmic membranes of *T. brucei* (Danson et al., 1987) and *lpd* mutant *E. coli* (Richarme, 1989) are not a component of ketoacid dehydrogenase but are

meant for uptake of monosaccharides in the cell. Such a cytoplasmic localization of the enzyme in *H. armigera* was not observed in the present studies. The mammalian PDC and KGDC are known to be located in the mitochondrial matrix space in association with the inner membrane (Maas and Bisswanger, 1990).

Biochemical documentation of the catalytic properties and the behavior of the multienzyme complexes are necessary to understand their role in metabolism. These studies will help to link the evolutionary relationships. DHLDH has been suggested by others as a target for antitrypanosomal and antibacterial drug design, as it is positioned at a critical branch point in metabolism (Williams, 1992). Arsenical drugs such as melarsoprol (Friedheim, 1994), melarsen oxide (Fairlamb et al., 1992; Milord et al., 1992) have been used for the treatment of African trypanosomiasis caused by trypanosome. These drugs mainly target oxidoreductase enzymes such as trypanothione reductase and lipoamide dehydrogenase. In the present study we observed that some of the arsenical compounds are potent inhibitors of Ha-DHLDH (Table 4.2). Therefore, it will be quite interesting to synthesize compounds targeting metabolic enzymes for the control of phytophagous insect pests such as *H. armigera*, since this insect has developed high level of resistance to commonly used insecticides. Studies of adult DLDH-deficient mice have suggested that a partial decrease of DLDH, which is sufficient to diminish activity of its associated enzyme complexes (Johnson et al., 1997), results in an elevated level of susceptibility to chemical neurotoxicity (Klivenyi et al., 2004). In view of the developed resistance of *H. armigera* to insecticides, evaluation of the specific inhibitors of the larvae may be worthwhile to overcome the insecticide resistance in the larvae, through newer mode of actions.

Because of the amenability of insects to adapt environment and develop resistance to several insecticides, biochemical properties of the enzymes involved in metabolism, such as DHLDH, could be helpful to develop insecticidal molecules for the control of *H. armigera*, with a different mode of action.

Chapter V

Detection of Pgp ATPase in the mitochondria of insecticide-resistant strain of Helicoverpa armigera

5.1. INTRODUCTION

Insecticides have been used for pest management worldwide. Because of high population build up and severe crop losses, insecticides have been used on a large-scale for controlling this cotton bollworm. *H. armigera* has developed high levels of resistance to organophosphates, carbamates, synthetic pyrethroids and organochlorine insecticides (Kranthi et al., 2001; Srinivas et al., 2004). Australian *H. armigera* population has also developed resistance (275-fold) to *B. thuringiensis* endotoxin Cry1Ac deployed in transgenic cotton (Gunning et al., 2005). Insecticide resistance in *H. armigera* is due to the combined effects of insensitivity of acetylcholine esterase to insecticides, expression of higher levels of esterases, phosphatases and a specific protein called p-glycoprotein ATPase (Srinivas et al., 2004).

Insects exhibiting resistance to one group of insecticides generally develop resistance to other classes of insecticides, a phenomenon often referred to as cross-resistance. This is similar to multidrug resistance (MDR) in cancer cells, whereby resistance to one drug is accompanied by simultaneous resistance to a variety of structurally unrelated compounds (Lanning et al., 1996). MDR in mammals is associated with over-expression of plasma membrane proteins that belong to the ATP-binding cassette (ABC) family (Leslie et al., 2005). The Pgp functions in ATP-driven efflux of drugs from the cell, and is thought to be an important cause of failure of cancer chemotherapy (Gottesman, 2002). Similar ABC proteins have been implicated in the resistance of many organisms to a vast and chemically diverse range of toxic molecules, and this type of resistance has been observed throughout the course of evolution (Prasad et al., 1996). The role of Pgp-like proteins in insects merits a study because such transporters may contribute to insecticide resistance. A few studies have

examined the possible role of Pgp-like proteins in the malphigian tubules of insects (Murray et al., 1994). A Pgp homolog was identified as a nicotine pump in the blood brain barrier of tobacco budworm, *Manduca sexta* L (Murray et al., 1994), and shown to maintain a decreased level of nicotine in the brain, thereby protecting the tobacco hornworm from toxicity to the central nervous system. Organochlorine and organophosphorus pesticides such as chlorpyrifos have been reported to bind to Pgp, and exposure to such compounds increases MDR1 gene expression (Bain and LeBlanc, 1996; Lanning et al., 1996). Exposure to the Pgp modulator verapamil increases the toxicity of ivermectin in chironomids (Podsiadlowski et al., 1998) and the toxicity of cypermethrin, ivermectin, and endosulfan in mosquitoes (Buss et al., 2002). Although Pgp is primarily located in the plasma membrane, it has also been detected in the nucleus (Baldini et al., 1995), golgi apparatus (Molinari et al., 1994) and in mitochondria (Munteanu et al., 2006; Solazzo et al., 2006).

5.2. MATERIAL AND METHODS

5.2.1. Glassware cleaning

Glassware cleaning procedure was similar to those as described in chapter II under the section 2.2.1.

5.2.2. Chemicals

Bovine serum albumin (BSA), ADP, ATP, FCCP (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone) were purchased from Sigma Aldrich (Mumbai, India). Sucrose was purchased from Qualigens (Mumbai, India), Tetramethylrosamine (TMR) from Molecular Probes (Eugene, OR, USA) and methylparathion (99.3%) and carbofuran (99%) from Pesticide Analysis Laboratory, Gulbarga, India. The other chemicals used in these studies were of analytical grade.

5.2.3. Insects

Insecticide-susceptible and resistant strains of *H. armigera* larvae were obtained from the insect rearing laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. The larvae were reared on a chickpea based semi-synthetic diet under laboratory conditions at 27 ± 1 ˚C, 65 ± 5% RH, and 12 h photoperiod (Armes et al., 1992). Preparation of artificial diet and rearing of *H. armigera* larvae is described in chapter II under the section 2.2.3.

5.2.4. Isolation of mitochondria

H. armigera larvae were starved for 3 h for the isolation of mitochondria. The mitochondria were isolated from the fourth- and fifth-instar larvae, and the experimental details are given in chapter II under the section 2.2.4.

5.2.5. Protein estimation

Protein concentration was determined by Lowry's method (1951) using BSA as a standard.

5.2.6. Mitochondrial respiration

Polarography determination of oxidative phosphorylation was made in oxygraph fitted with a Clark type oxygen electrode. Respiratory parameters were measured in presence or absence of insecticide for the substrates [succinate (10 mM)]. The experimental details were described in chapter II under the section 2.2.7.

5.2.7. Pgp ATPase activity

The ATPase activity was determined by quantitating the release of inorganic phosphate from ATP (Borgnia et al., 1996). An aliquot of mitochondrial extract was incubated in 1 ml of ATPase assay medium (containing 2.5 mM ATP; 75 mM KCl; 5

mM $MgCl_2$; 0.5 mM EGTA; 2 mM ouabain; 3 mM sodium azide; 50mM Tris–HCl, pH 7.4) for 15 min at 37 °C. The reaction was terminated by the addition of 2 ml ice-cold stopping medium [0.2% (w/v) ammonium molybdate, 0.9% SDS, 2.3% trichloroacetic acid, 1.3% (w/v) sulfuric acid, and freshly prepared 1% (w/v) ascorbic acid]. After 15 min incubation at room temperature, the released phosphate was quantitated colorimetrically at 660 nm.

5.2.8. Substrate specificity

Substrate specificity was tested using AMP, ADP and ATP for the release of inorganic phosphate with the mitochondrial Pgp ATPase.

5.2.9. Electrophoresis

The mitochondrial fraction (200 mg/ml) was solubilized in 1% 3-[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS) in 50 mM potassium phosphate (pH 7.2) and kept on ice for 10 min. The sample was spun at 12,000 x g for 1 h to eliminate mitochondrial fragments. The supernatant was subjected to SDS-PAGE on 7.5% polyacrylamide gels according to Laemmli (1970). Glycoprotein staining was carried out with periodic acid-Schiff reagent using the procedure described by Gerard (1990).

5.2.10. Western blotting

The presence of a Pgp-like protein in the isolated mitochondria from the insecticide resistant larvae was confirmed as described previously (Aurade et al., 2010), using Western blotting with C219 antibodies, which were directed at the C-terminal NBD of mammalian Pgp.

5.2.11. Detection of cytochrome c release

Mitochondria (1 mg protein) isolated from insecticide-susceptible and resistant larvae were incubated in 50 mM phosphate buffer, pH 7.2, containing 500 µM of insecticide

(methylparathion and carbofuran) for 1 h in the presence of 0.1 mM Ca^{2+}. The experimental details are described in chapter III, section 3.2.9.

5.2.12. Functional assay of mitochondrial Pgp by spectroflurometer

Mitochondrial fraction was prepared from insecticide-susceptible and -resistant *H. armigera* larvae as described above. The mitochondria were suspended in an isolation media on ice till the experiment was performed. Rhodamine a known substrate for Pgp was used as an indicator of Pgp activity (Kim et al., 1998). The rhodamine derivative, TMR was used to evaluate the uptake and efflux function of mitochondrial Pgp in intact mitochondria. All the fluorometric experiments were performed at the room temperature using Cary Varian Eclipse fluorescence spectrophotometer in kinetic mode. Excitation was carried out at 550 nm (slit width, 5 nm) and the fluorescence emission was monitored continuously at 575 nm (slit width, 5 nm). The reaction mixture contains 50 mM sucrose, 0.02% BSA, 10 mM succinate, 200 nM TMR and 200 mg of mitochondrial protein. The transport of dye was measured by facilitating the Pgp activity in the presence of 2.5 mM ATP, or by inhibiting Pgp in the presence of 1 mM sodium vanadate (inhibitor of Pgp) in isolated mitochondria from the insecticide-resistant larvae.

5.2.13. Mitochondrial membrane potential ($\Delta\psi$)

The transmembrane potential was evaluated in isolated mitochondria according to the modified method of Braguini et al., (2004), using rhodamine B, in the same reaction mixture used above for measuring the Pgp assay. Fluoresence was measured in Cary Varian Eclipse fluorescence spectrophotometer in kinetic mode. The excitation and emission wavelengths being 550 and 575 nm, respectively, with a slit width of 5.0 for excitation and emission wavelengths. Mitochondria were energized with sodium succinate (5 mM), and

FCCP (10 µM) (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone) was used as uncoupler to diminish membrane potential. Membrane potential was calculated according to the formula, $\Delta F = F\text{-}F_0/F_0 \times 100$; where, F is the fluorescence intensity at the point of stimulus, and F_0 is the baseline fluorescence intensity (Joshi and Bakowska, 2011).

5.2.14. Statistical analysis

Data were subjected to one way analysis of variance (ANOVA) as described in chapter II, section 2.2.15.

5.3. RESULTS

5.3.1. Mitochondrial respiration

Amount of oxygen consumed was 63.24 ± 5.23 nmoles/min/mg protein for the control mitochondria. At 100 µM concentration, methylparathion inhibited 77.78 and 39.40% of oxygen consumed in mitochondria isolated from insecticide-susceptible and -resistant larvae compared to control mitochondria, respectively. At this concentration, it inhibited 74.62% of the oxygen consumed by mitochondria isolated from insecticide-resistant larvae when sodium vanadate was included in the oxygen consumption reaction mixture. The inhibition was similar to that observed in mitochondria isolated from insecticide-susceptible larvae (Fig. 5.1). Carbofuran induced similar effects in insecticide-susceptible and resistant larvae.

5.3.2. Pgp ATPase activity and substrate specificity

Methylparathion and carbofuran induced the activity of Pgp at lower concentrations, but inhibited the activity at higher concentrations *in vitro*. At 40 µM methylparathion and carbofuran induced the Pgp ATPase activity by 52 and 48%, respectively (Fig. 5.2).

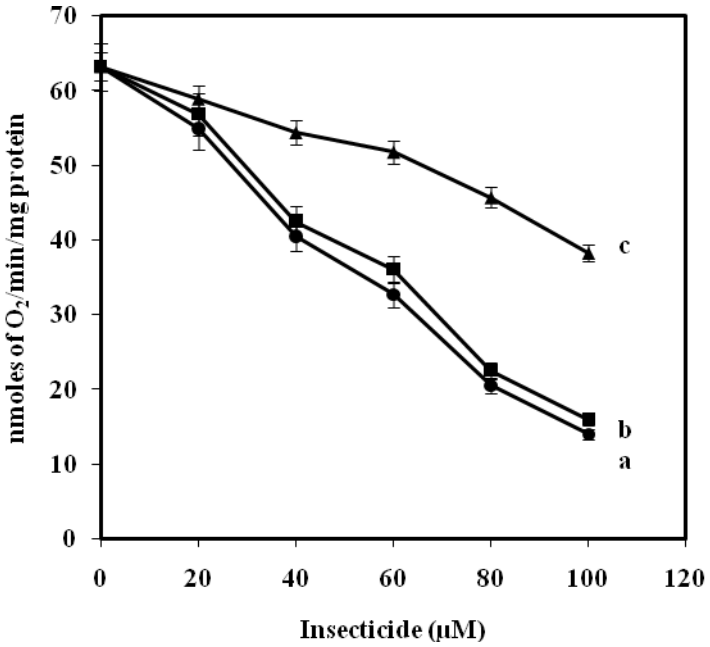


Fig. 5.1. Effect of methyl-parathion on mitochondrial respiration when succinate was used ase oxidizable substrate. The insecticide was incubated with mitochondria for 2 min prior to addition of succinate. Oxygen uptake in mitochondria isolated from: insecticide-susceptible larvae (a), insecticide-resistant larvae in presence of sodium vandate (b), and mitochondria isolated from insecticide-resistant larvae in absence of sodium vandate (c). The values are based on three individual experiments.

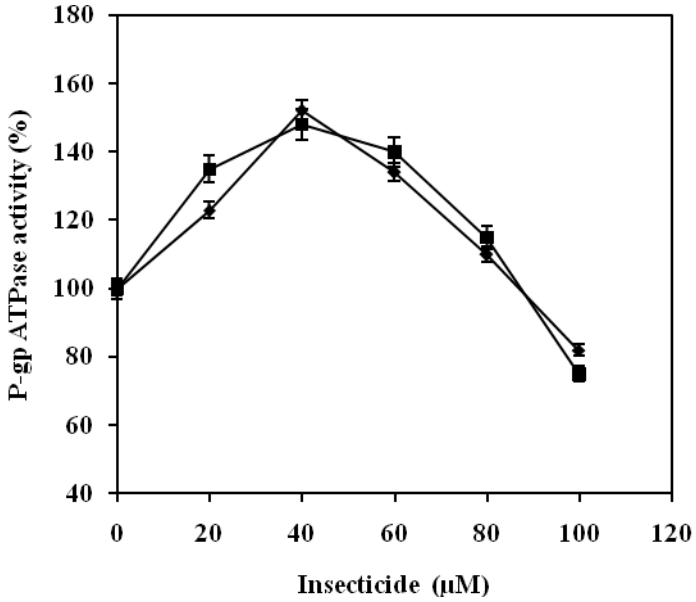


Fig. 5.2. Effect of methyl-parathion (♦) and carbofuran (■) on mitochondrial Pgp ATPase activity. The graph shows percentage stimulation compared to the control (100%, in the absence of pesticides). Data points represent the means based on three determinations.

Substrate specificity tested in the direction of hydrolysis of adenine nucleotides indicated that the enzyme was highly specific to ATP as substrate, and did not use ADP and AMP.

5.3.3. Electrophoresis and western blotting

Presence of Pgp in the mitochondria was detected by SDS-PAGE and confirmed by Western blotting using C219 antibodies, which are specific to the nucleotide binding domain of Pgp (Fig. 5.3). Molecular weight of Pgp was found to be 150 kDa as determined by SDS PAGE.

5.3.4. Detection of cytochrome c release

Methylparathion and carbofuran induced the efflux of cytochrome c when incubated with isolated mitochondria from insecticide-susceptible larvae in the presence of Ca^{2+}, it was found to be 1.76 and 1.56 nmol/mg protein of cytochrome c, respectively, in the supernatant; whereas, in insecticide-resistant larvae, a non-significant amount of cytochrome c was detected (Fig. 5.4).

5.3.5. Functional assay of mitochondrial Pgp by spectroflurometer

Accumulation and efflux studies were carried out using TMR. Addition of 2.5 mM ATP to the reaction mixture containing freshly isolated intact mitochondria, which were earlier incubated with 200 nM TMR, led to a rapid increase in TMR fluorescence reaching a steady-state level. In presence of insecticides, the efflux of TMR was reduced, and the decrease in efflux of TMR in presence of insecticide occurred in a dose-dependent manner for both the insecticides, methylparathion and carbofuran. Data is shown only for methylparathion (Fig. 5.5 (i), B,C,D). The initial rate of TMR transport declined in a dose-dependent fashion with an increase in insecticide concentration (Fig. 5.5 (ii)), with 30 - 40% inhibition at 50 µM. Thus, insecticide inhibited the net rate of transport of TMR, and

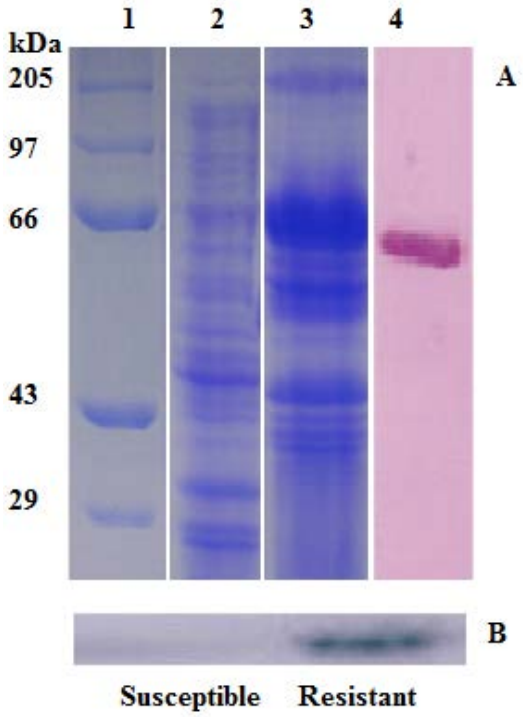


Fig. 5.3. SDS-PAGE (7.5%) profile for detection of mitochondrial Pgp ATPase from *H. armigera*. (A) The gel was stained with Coomassie blue, lane 1, molecular weight markers (29 – 205 kDa); lane 2, Triton-dissolved mitochondrial extract (100 μg); lane 3, Partially purified mitochondrial extract; lane 4, glycosylation of Ha-Pgp; (B) detection of Pgp in the mitochondrial fraction using C219 antibodies.

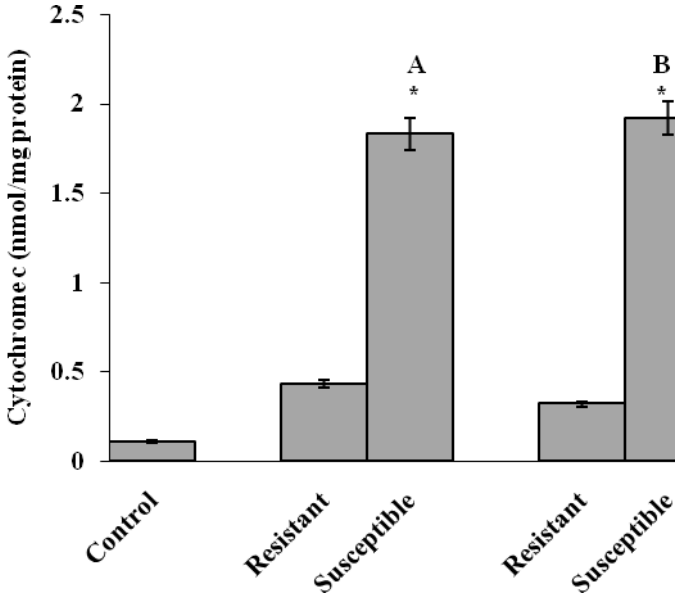


Fig. 5.4. Detection of cytochrome c release. Cytochrome c release was estimated by incubating mitochondria (1 mg protein) isolated from insecticide-susceptible and -resistant larvae in the presence of methylparathion (A) and carbofuran (B). The values are the means of three individual experiments.

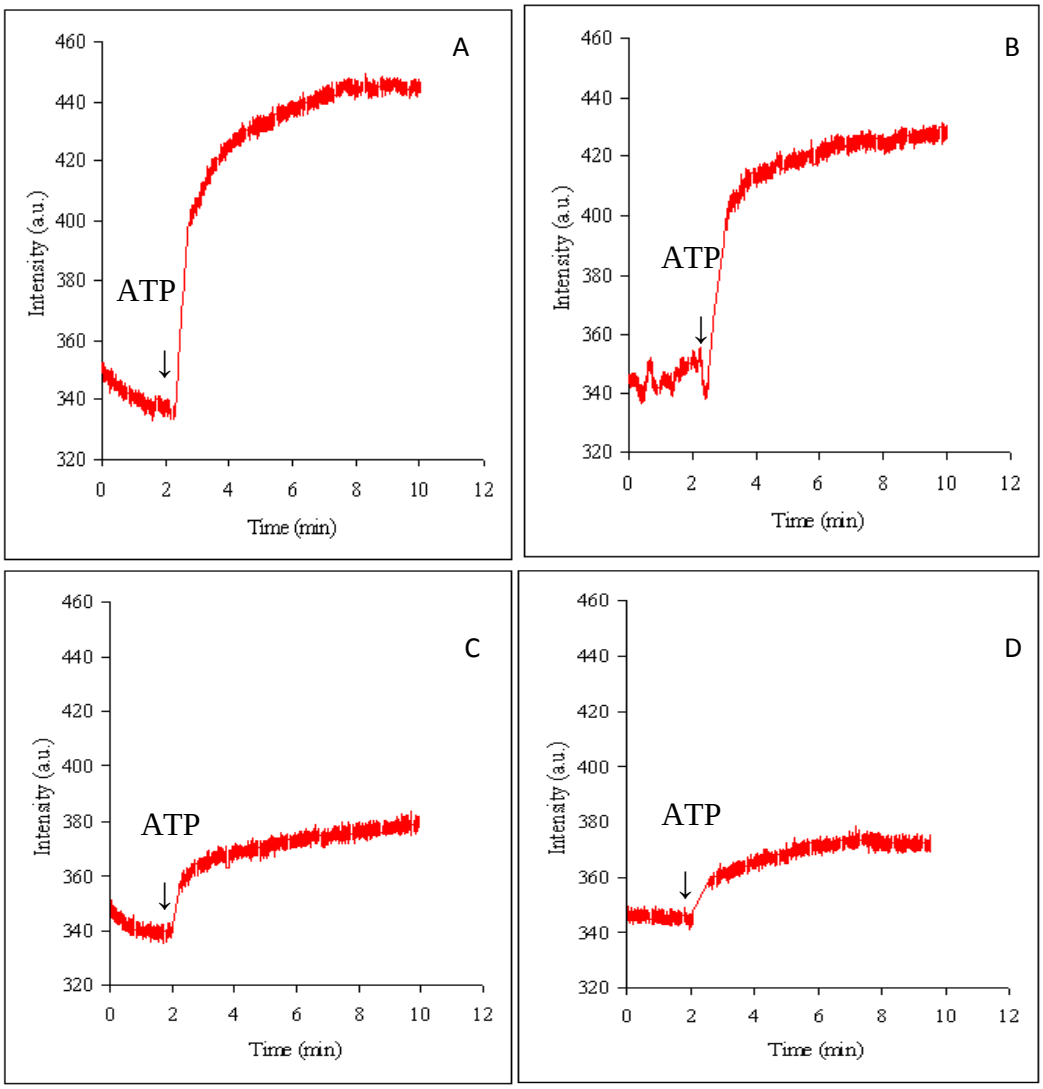


Fig. 5.5 (i). Efflux studies of TMR in intact mitochondria. A: Control, B: 10 µM methyl-parathion, C: 30 µM methyl-parathion, and D: 50 µM methyl-parathion.

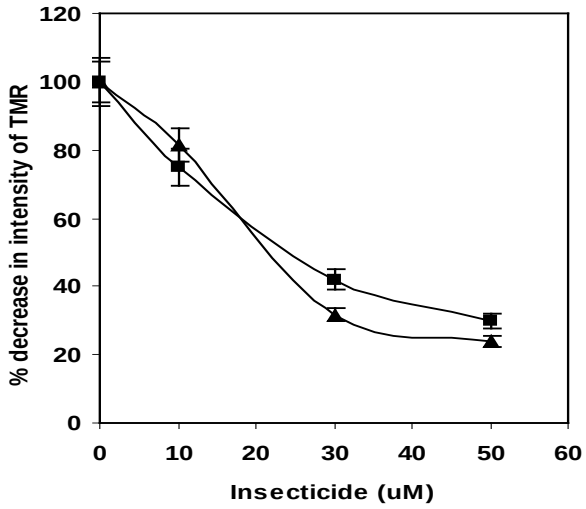


Fig. 5.5 (ii) Inhibition of movement of TMR in presence of methyl-parathion (▲) and carbofruan (■) in intact mitochondria isolated from insecticide-resistant larvae. The values are based on three individual experiments.

both the insecticides competed effectively with TMR for the transport via Pgp. Measurement of fluorescence intensity of TMR when incubated with mitochondria isolated from insecticide- susceptible larvae in absence of ATP indicated that, the intensity of TMR decreased with time, indicating accumulation of TMR inside the mitochondria. TMR also accumulated in mitochondria isolated from insecticide-resistant larvae in presence of sodium vanadate, an inhibitor of Pgp (Fig. 5.6).

5.3.6. Effect of insecticides on the membrane potential (Δψ) in isolated intact mitochondria

Methylparathion and carbofuran did not affected the membrane potential at lower concentrations (< 50 μM), but impaired the development of Δψ in mitochondria isolated from insecticide-resistant larvae at higher concentrations (> 50 μM) (Fig. 5.7) (Table 5.1).

5.4. DISCUSSION

Insecticide resistance in *H. armigera* is characterized by several features, including over-expression of Pgp in cell membranes (Srinivas et al., 2004). Pgp is an ABC protein that functions by pumping out drugs and other compounds from the cells. Pgp is provides multidrug resistance in cancer cell (Ueda et al., 1987).

The mitochondrial Pgp ATPase has been reported from several cancerous cell lines (Munteanu et al., 2006; Solazzo et al., 2006). In the present studies, we report for the first time the presence of Pgp from the mitochondria of *H. armigera* and demonstrate its transport function in intact mitochondria using spectrofluorometer in kinetic mode. The Pgp ATPase activity measured *in vitro* was enhanced in the presence of lower concentrations, but inhibited in the presence of high concentrations of insecticides. Presence of Pgp in the

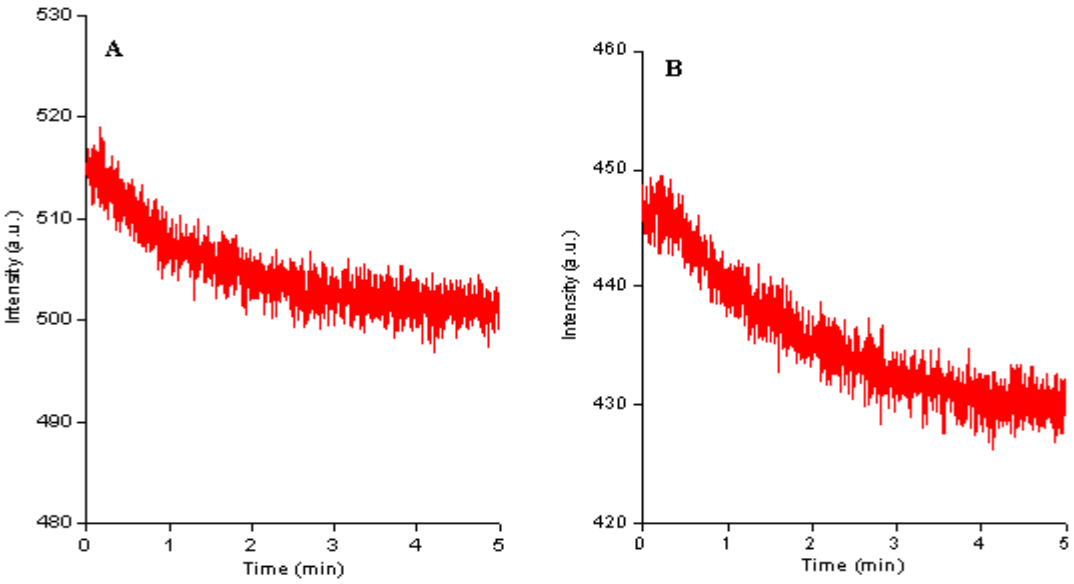


Fig. 5.6. Accumulation of TMR in absence of ATP in intact mitochondria isolated from (A) insecticide-susceptible larvae, and (B) insecticide-resistant larvae, in presence of sodium vanadate. The means are based on three individual experiments.

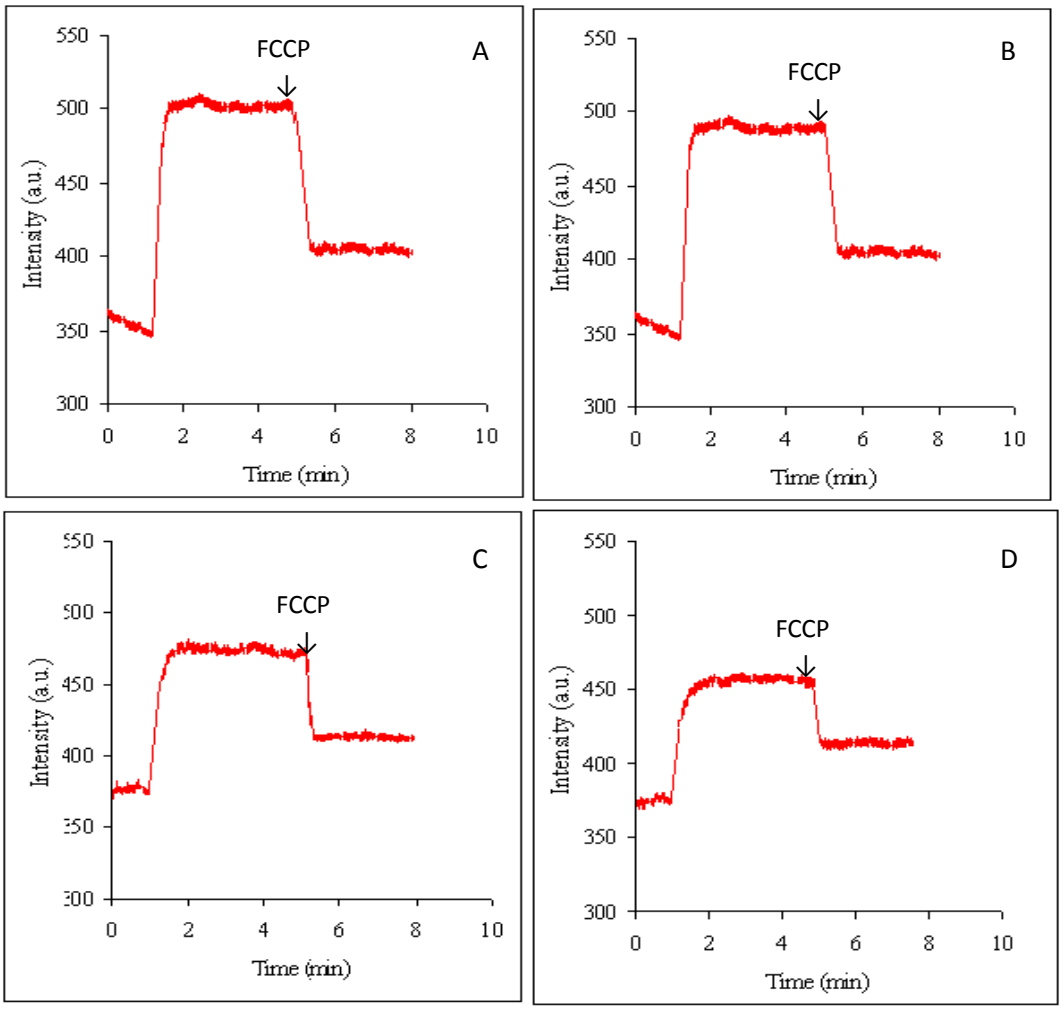


Fig. 5.7. Membrane potential of the mitochondria isolated from insecticide-resistant larvae: (A) control, (B) 50 µM methyl-parathion, (C) 100 µM methyl-parathion, and (D) 150 µM methyl-parathion. The means are based on three individual experiments.

Table 5.1: Mitochondrial membrane potential ($\Delta\psi_m$)

Insecticide	$\Delta\psi_m$ in presence of methylparathion	$\Delta\psi_m$ in presence of carbofuran
Control	25	25
50 µM	20	18.46
100 µM	18.75	13.98
150 µM	12.5	10.48

mitochondria was confirmed in mitochondrial fraction using periodic staining in SDS PAGE and C219 antibodies using western blotting on PVDF membranes (Fig. 5.3). Similar reports have been reported from cancerous cells lines (Solazzo et al., 2006). Rate of oxygen consumption in mitochondria isolated from insecticide-susceptible larvae decreased in the presence of insecticides in a dose-dependent manner. Oxygen consumption in mitochondria isolated from the insecticide-resistant larvae withstood the inhibition of oxygen consumed at lower concentrations (< 60 µM) (Fig. 5.1). This might be due to the presence of Pgp, which could extrude the insecticide and protects the respiratory enzyme complexes present in the inner membrane of mitochondria from the toxic effect of insecticides. In presence of sodium vanadate, an inhibitor of Pgp, oxygen consumption in the mitochondria isolated from insecticide-resistant larvae was similar to the mitochondria isolated from insecticide-susceptible larvae. As such, sodium vanadate had no effect on the rate of oxygen consumption.

Cytochrome c was released into the supernatant when mitochondria of insecticide-susceptible larvae was incubated with the insecticides, but not in the mitochondria from the insecticide-resistant larvae (Fig. 5.4). This might be due to the over-expression of Pgp in the mitochondria, which prevented the release of cytochrome c. These results are in accordance with studies on mitochondria from cancerous cells, wherein, MDR cells block the release of cytochrome c from mitochondria into cytosol (Solazzo et al., 2006). TMR has been used as a substrate for studying the transport function of Pgp (Lu et al., 2001). In the present studies, unlike the confocal imaging, Pgp mediated transport was evaluated using fluorescent probe TMR in intact mitochondria in spectrofluorometer. TMR accumulated in mitochondria isolated from insecticide-susceptible larvae in absence of ATP, and the similar

effects were observed with mitochondria isolated from the insecticide-resistant larvae in presence of vanadate, an inhibitor of Pgp (Fig. 5.6). In presence of ATP, the dye is effluxed out of the mitochondria isolated from insecticide-resistant larvae as evidenced by increase in fluorescence intensity of TMR (Fig. 5.5 (i)). No increase in TMR fluorescence was observed in the presence of non-hydrolysable ATP analogs. This indicated that the process of TMR transport requires ATP hydrolysis and functionally active Pgp. Prior incubation of mitochondria isolated from insecticide-resistant larvae with insecticide has shown a dose-dependent decrease in the efflux of dye, suggesting that the insecticides competed with TMR, and inhibited the net transport of the dye (Fig. 5.5 (ii)).

These results indicated that mitochondrial membrane contains Pgp, which is functionally active and involved in efflux of insecticides and is oriented in a direction which pumps out the insecticides into cytosol. Orientation of Pgp in the mitochondrial membrane is still controversial, but some reports suggested that Pgp is oriented in mitochondria of MDR cells outside-in, leading to accumulation of drugs (Munteanu et al., 2006); and inside-out- effluxing out the drugs from the cell organelle into cytosol (Solazzo et al., 2006). However, those studies were carried out using confocal microscopy, where accumulation of the dye inside the cell organelle was measured. In the present studies, measurement of movement of TMR was studied in intact mitochondria in a kinetic mode in spectrofluorometer. Accumulation of the dye was observed in insecticide-susceptible and -resistant larvae in absence of ATP. In presence of ATP, the active transport of the dye increased in intensity, indicating the efflux of dye in the insecticide-resistant larvae, and the process was inhibited in the presence of sodium vanadate, an inhibitor of Pgp. These experiments clearly demonstrated that the orientation of Pgp is inside-out, which extrudes

the insecticidal molecules from the mitochondria. Resistance to inhibition of oxygen consumption observed in the mitochondria isolated from insecticide-resistant larvae can now be explained based on results obtained from the efflux studies of TMR in spectrofluorometer. Ling et al., (2012) observed that mitochondrial Pgp is over-expressed in human hepatoma cell line depleted of mitochondrial DNA, which facilitates the exclusion of chemotherapeutics from the mitochondria into cytosol.

In presence of insecticides, the membrane potential progressively decreased in a dose-dependent manner in case of mitochondria isolated from the insecticide-susceptible larvae. These results are in accordance with those of Braguini et al., (2004). Membrane potential was not affected with lower concentrations of the insecticides in the mitochondria isolated from the insecticide-resistant larvae, but at higher concentration of the insecticides, $\Delta\psi$ decreased in a dose-dependent manner (Fig. 5.7). At lower concentrations of the insecticide, Pgp ATPase activity was induced, and hence, the insecticide was pumped out without affecting the membrane potential. The mitochondrial membrane potential quite high in cancerous cells (Gogvadze et al., 2008). In contrast, the $\Delta\psi$ was found to be similar in the mitochondria isolated from the insecticide-susceptible and -resistant larvae (Table 5.1). Therefore, the mitochondria of the insecticide-resistant *H. armigera* larvae possess unique characteristics similar to cancerous cells, including over-expression of functionally active Pgp, but with normal mitochondrial characteristics such as generation of ATP and normal mitochondrial $\Delta\psi$ which is contrary to cancer cells. This could be one of the adaptations in insects to provide protection to the cell organelle from insecticides, which in turn results in insect resistance to insecticides. Such an adaptation might be acquired independently by insects during long-term exposure to insecticides.

In conclusion, the present studies demonstrated that Pgp is expressed in the mitochondrial membrane of insecticide-resistant *H. armigera* larvae. Pgp is functionally active and works like a pump to extrude insecticidal molecules from the mitochondria. This mechanism is likely to be involved in the protection of mitochondrial DNA and the components of electron transport chain from damage due to the insecticides in insecticide-resistant larvae of *H. armigera*.

Chapter VI

Characterization of storage proteins from the hemolymph of Helicoverpa armigera

6.1. INTRODUCTION

Storage proteins, such as arylophorin, are synthesized from the fat body of a wide range of lepidopteran and dipteran larvae and also in other insect orders. They are the major proteins in the hemolymph. Storage proteins are taken up by the fat body shortly before pupation and stored in protein granules. The central function of the proteins seems to be a storage pool of the amino acid resources for complete development of the adult. There are at least two kinds of storage proteins in lepidoptera. They form loose clusters on a bivariate plot of the proportion of aromatic amino acid (tyrosine plus phenylalanine) versus methionine (Telfer and Kunkel, 1991). Arylphorin is rich in aromatic amino acids, and the second protein is referred to as methionine-rich storage protein.

Insects prepare for the synthetic demands of molting, metamorphosis and reproduction by accumulating hexamerins in their hemolymph and fat body. First to be described were two soluble storage hexamerins isolated from larvae of Calliphora erythrocephala (Munn and Greville, 1969). Two or more hexamerins were later reported in the hemolymph and fat body of many other insects, with some lepidopterans having as many as four. Hexamerins occurring within a single species can differ in amino acid composition, stage of synthesis, distribution between hemolymph and fat body, timing of clearance from the hemolymph, and antigenic reactivity (Kanost et al., 1990; Telfer and Kunkle, 1991). Storage proteins have mostly been studied in Diptera and Lepidoptera, but more recently also in other insect orders. Storage proteins have also been found in other insect orders; arylphorins as well as other hexamerins were described in bees (Shipman et al., 1987), ants (Wheeler and Martinez, 1995), beetles (DeKort and Koopmanschap, 1994), but also in the hemimetabolous locusts (DeKort and Koopmanschap, 1987) and cockroaches (Duhamel and Kunkel, 1983; Jamroz et al., 1996).

6.2. MATERIAL AND METHODS

6.2.1. Glassware cleaning

Glassware cleaning procedure was similar to those as described in chapter II under the section 2.2.1.

6.2.2. Chemicals

DEAE-cellulose, Coomassie brilliant blue R and sudan black were purchased from Sigma Aldrich (Mumbai, India). Tris-HCl and EDTA were purchased from Himedia (Mumbai, India), while the other chemicals used were of analytical grade.

6.2.3. Insect culture

Larvae of *H. armigera* were obtained from the insect rearing laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. The larvae were reared on a chickpea based semi-synthetic diet under laboratory conditions as described earlier in Chapter II under the section 2.2.3.

6.2.4. Collection of hemolymph

Hemolymph was collected from the *H. armigera* larvae according to Charalamibidis et al., (1995). Hemolymph samples (approx. 10 ml from 3 days old pupae) were dialyzed in 250 ml of buffer (50 mM Tris-HCl and 1 mM EDTA pH *7*) for approximately 5 h. and then centrifuged again at 7,800 g.

6.2.5. Purification of storage protein

Ammonium sulfate was added to 50 ml of hemolymph extracted from the third- and fourth-instar larvae (1,500 mg of protein) to give 40% saturation. The solution was kept for overnight stirring at 4 ºC, and the precipitate was removed by centrifugation for 30 min at 10,000 g, and the supernatant was brought to 60% ammonium sulfate saturation. After

overnight stirring at 4 ºC, salt precipitated proteins were collected by centrifugation for 30 min at 10,000 g, and dissolved in buffer 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, and dialyzed for 18 h against the preceding buffer at 4 ºC. This pool was applied to DEAE cellulose (2 x 10 cm) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA. After the column was washed with 100 ml of the same buffer, proteins were eluted with a step gradient of 0 to 0.5 M NaCl and the fractions were collected each of 3 ml. The eluant was monitored at 280 nm.

6.2.6. Molecular weight determination

SDS-PAGE was carried out in 10% polyacrylamide in non-reducing conditions according to the method of Laemmli (1970). Native-PAGE was performed in 5 - 8% gradient polyacrylamide gels. The gels were stained after electrophoresis with Coomassie brilliant blue R and sudan black seperately (Slovak and Repka, 1993).

6.2.7. Amino acid composition

Purified protein was subjected to SDS-PAGE. Areas of storage protein were excised and sent for analysis of amino acid composition at HSC Advanced Protein Technology Center, Department of Structural Biology and Biochemistry, Toronto, Canada.

6.2.8. LCMS sequence of storage protein

For determination of LCMS sequence, purified protein was subjected to SDS-PAGE. The storage protein was detected in gel by sudan black staining. Areas of brown stain were excised and sent for sequence analysis at HSC Advanced Protein Technology Center, Department of Structural Biology and Biochemistry, Toronto, Canada.

6.2.9. Phylogenetic tree of the storage protein

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.10543165 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 79 positions in the final dataset. Cladistic analysis was performed using the programs provided with MEGA 4 (Tamura et al., 2007).

6.3. RESULTS

6.3.1. Identification of storage protein

Total hemolymph proteins were screened by SDS-PAGE in order to identify storage proteins. On the basis of Coomassie blue staining, these proteins increased in relative abundance in hemolymph throughout the final instars. Sudan black staining was used to stain the lipopolypeptides.

6.3.2. Purification of storage protein

Hemolymph from the late instars served as the starting material for the purification of storage proteins. An indispensible first step was fractionation by ammonium sulphate. At first, the dialyzed hemolymph was saturated with 40%, later the precipitation was raised to 60%. The precipated proteins were subjected to anion exchange chromatography. The

storage protein was eluted as a single peak from DEAE-cellulose, and gave a single band when analyzed by SDS-PAGE. From 1,500 mg of total protein, 7.35 mg of purified protein was obtained with 24.4% recovery (Table 6.1).

6.3.3. Molecular weight determination

Subunit molecular weight of purified storage protein was estimated to be 66 kDa (Fig. 6.1). The native molecular weight of the protein was determined to be 400 kDa.

6.3.4. Alignment of LCMS sequence

An initial alignment of LCMS sequence was generated by the program Clustal X software package. For multiple sequence alignment, the complete sequences for different organisms were obtained from NCBI website, and the sequence was manually corrected and aligned by NCBI BLAST (Fig. 6.2). Upon the sequence homology, the protein was identified as arylphorin.

6.3.5. Phylogenetic tree of the arylphorin of *H. armigera*

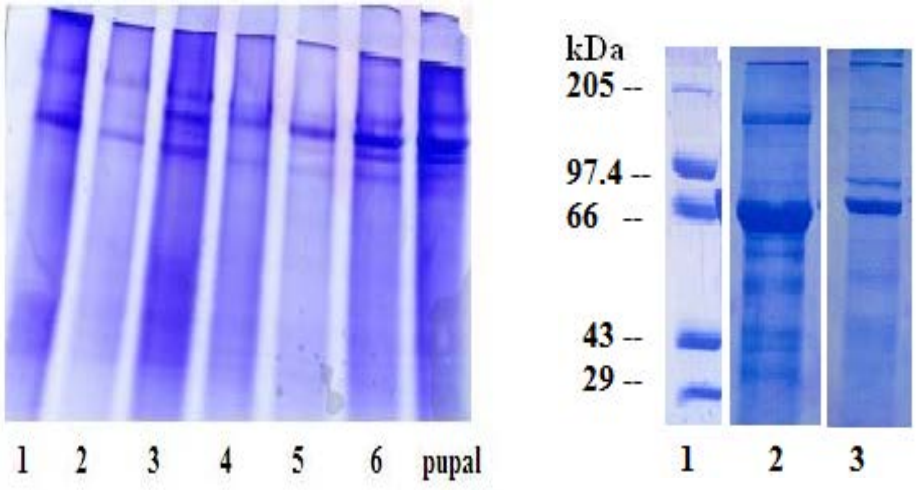
Phylogenetic analysis showed that the isolate had maximum similarity (more than 99%) with *Spodoptera litura* and *Manduca sexta* among the eight taxas (Fig. 6.3).

6.3.6. Amino acid composition

Aminoacid composition obtained from *H. amigera* is shown at Table 2, along with those of the *B. mori* and *H. cecropia*. The protein was characterized by an higher levels of glutamate, phenylalanine and tyrosine (Table 6.2).

Table 6.1: Purification of storage proteins:

S. NO.	Fraction	Total protein content (mg/ml)	Recovery (%)
1.	Crude hemolymph	30.12 ± 3.54	100
2.	60% $(NH_4)_2SO_4$ ppt	27.84 ± 1.23	92.4
3.	DEAE cellulose	7.35 ± 0.74	24.4



A B

Fig. 6.1. (A**)** Native gel electrophoresis of hemolymph proteins from 1 to 6 (lane 1 to 6) instars and pupae of *H. armigera. (B)* SDS PAGE of hemolymph proteins of *H. armigera* (lane 1, molecular weight markers; lane 2, 60% ammonium sulphate precipitated hemolymph extract; and lane 3, purified protein).

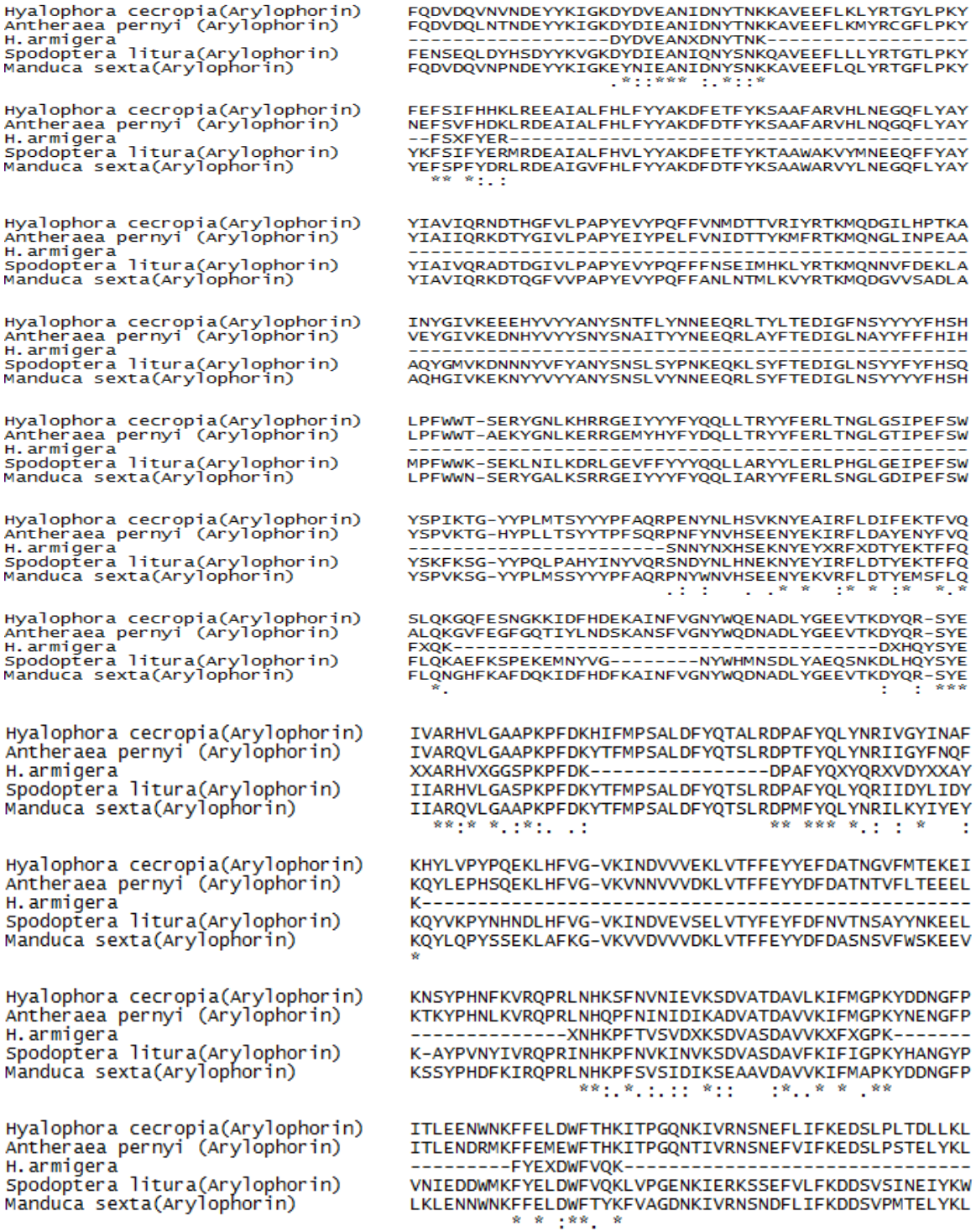


Fig. 6.2. Multiple deduced amino acid sequence alignment of obtained sequence of *H. armigera* with *Hyalophora cercopia, Antheraea pernyi, Spodoptera lutira and Manduca sexta.*

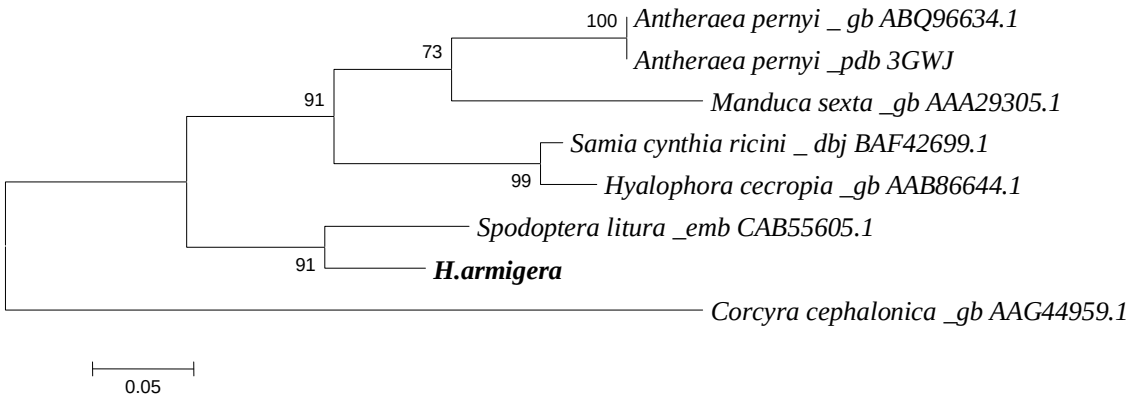


Fig. 6.3. Phylogenetic tree for the arylphorin protein of *H. armigera.*

Table 6.2: Amino acid composition of the arylophorin of *H. armigera* compared with the SP-2 (arylphorin) of *B. mori* and *H. cecropia*.

Amino acid	*H. armigera*	*B. mori* (Tojo et al., 1979)	*H. cecropia* (Tojo et al., 1978)
Cysteine	5	8	Trace
Aspartate	130	121	134
Threonine	53	45	55
Serine	46	40	49
Glutamate	110	125	94
Proline	56	61	19
Glycine	38	40	39
Alanine	48	54	36
Valine	64	61	75
Methinine	30	27	49
Isoleucine	37	38	52
Leucine	72	69	106
Tyrosine	102	89	68
Phenylalanine	100	98	62
Lysine	88	85	100
Histidine	10	13	9
Arginine	25	32	51
Tryptophan	--	--	--
Total	1014	1006	998

6.4. DISCUSSION

Storage proteins, such as arylophorin, are synthesized from the fat body of a wide range of lepidopteran and dipteran larvae and also in other insect orders. They are the major proteins in the hemolymph. Storage proteins are taken up by the fat body shortly before pupation and stored in protein granules. The central function of the proteins seems to be a storage pool of the amino acid resources for complete development of the adult. There are at least two kinds of storage proteins in lepidoptera. They form loose clusters on a bivariate plot of the proportion of aromatic amino acid (tyrosine plus phenylalanine) versus methionine (Telfer and Kunkel, 1991). Arylphorin is rich in aromatic amino acids, and the second protein is referred to as methionine-rich storage protein. All have molecular masses of nearly 500 kDa and are hexamers composed of approximately 80 kDa subunits. Arylphorins are a class of insect storage proteins that have an unusually high content of the aromatic amino acids phenylalanine and tyrosine (Telfer et al., 1983) and belong to the larger class of storage proteins known as the hexamerins. The arylphorins are high molecular weight complexes composed of six similar (or identical) subunits. They are related to the arthropod hemocyanins, and more distantly to the phenyloxidases (Burmester, 2002).

Storage proteins of *H. armigera* was obtained by ammonium sulphate fractionation and anion-exchange chromatography. Storage proteins purified from *B. mori* was done on the ammonium sulphate fractionation, cation exchange followed by anion exchange chromatography. Arylphorins have 2 - 10% (w/w) carbohydrate content consisting of mainly GlcNAc and Man in a 1:4±5 molar ratio (Telfer et al., 1983; Tojo and Yoshiga, 1993).

Subunit molecular weight of the arylphorin was found to be 66 kDa (Fig. 6.1). Hexamerin structure has generally been inferred from molecular weight estimates for native and dissociated forms of the proteins in polyacrylamide gel electrophoresis (PAGE) (Telfer 1991).The molecular properties of *H. armigera* are in agreement with those of the *B. mori*, where the native molecular weight was reported to be 500 kDa, with a subunit mass of approximately, 85 kDa. The storage proteins of four Diptera (*C. erythrocephala, C. stygia, L. cuprina and D. melanogaster*) also consists of subunits of 80-85 kDa, associated with either hexamers (mol. wt. 500 kDa) or trimers (250 kDa) (Munn et al., 1971; Thomson et al., 1976; Wolfe et al., 1977). The concentration of storage protein increase in the hemolymph from the first instar to the final instar of larval development. The concentrations of the storage proteins in the hemolymph change during development of insect larvae from the final instar to the larva-pupal ecdysis. Such changes are essentially similar to those of two storage proteins in *H. cecropia* (Tojo et al., 1978) and *B. mori* (Tojo et al., 1980). In *B. mori* it has been reported that the two storage proteins (SP-1 and SP-2) increase in the hemolymph from the final instar and reach maximal levels at time of spinning and then during the larval-pupal transformation decrease in the hemolymph (Tojo et al., 1980).

Amino acid composition showed that the protein was characterized by the presence of aromatic amino acids (Table 6.2). Munn et al., (1971) proposed that a high aromatic amino acid content is related to demands for these constituents during sclerotization of the cuticle. SP-2 (arylphorin) of *B. mori* has been reported to contain high quantity of tyrosine and phenylalanine (Tojo et al., 1980). Dipteran protein isolated from *Calliphora erythrocephala* (Munn and Greville, 1969) had both, aromatic and methionine high, and

given the name calliphorin. Calliphorin was shown to be synthesized by the fat body (Munn et al., 1969) and secreted into the hemolymph, beginning about half way through the larval growth period. Arylphorin purified was identified by data obtained by LCMS sequence. The deduced amino acid sequence was found to bore homology with the arylphorins of *Spodoptera litura, Manduca sexta, Hyalophora cecropia,* and *Antheraea pernyi* (Fig. 6.2).

In conclusion, arylphorin, a storage protein, one of the key in metamorphosis, reproduction and insect development, are purified, partially sequenced and its amino acid composition deduced. Now a days disruption of the regulation of storage proteins is a potential method to control this pest.

Summary

Helicoverpa armigera is a major pest of agricultural crops, and has been reported to attack more than 200 different species of plants including cotton, pigeonpea, chickpea, groundnut sorghum, maize, vegetables, fruit and forest trees. The lepidopteran larvae essentially are 'eating machines', and grow much faster than mammals and birds with a high rate of metabolic processes. This phenomenal growth occurs due to large midgut epithelium, which digests and absorbs the nutrients, and requires the presence of a complete mitochondrial system for oxidative phosphorylation to meet the energy demands of the insect undergoing metamorphosis through different larval instars.

Effect of methylparathion, carbofuran, and permethrin and fenvalarate was studied on oxidative phosphrylation and oxidative stress in *H. armigera.* ADP-stimulated respiration (state III) was inhibited by all the insecticides *in vitro* when succinate was used as the oxidizing substrate. The insecticides also inhibited the rate of oxygen consumption after ADP exhaustion (state IV). RCI and P/O ratios were also inhibited in a dose-dependent manner in isolated mitochondria with an exception of pyrethroids on P/O ratio. A clear inhibitory effect on succinate dehydrogenase is induced by methylparathion and carbofuran, whereas permethrin and fenvalarate inhibited both complexes I and II *in vitro.* Under *in vivo* conditions, state III respiration and state IV respiration decreased, affecting RCI. Insecticide exposure induced-lipid peroxidation, H_2O_2 content and LDH leak in a time- and dose-dependent manner, altered the level of antioxidant enzymes, leading to oxidative stress in cells, resulting in deleterious effects on the growth of insecticide-susceptible *H. armigera* larvae, along with the neurotoxic effects.

Salicylic acid (SA) and jasmonic acid (JA) are the cell signaling molecules produced in response to insect attack in plants. The effect of these signaling molecules was

investigated on the oxidative phosphorylation and oxidative stress of *H. armigera*. SA significantly inhibited the state III and state IV respiration, respiratory control index (RCI), respiratory complexes I and II, induced mitochondrial swelling, and cytochrome c release *in vitro*. Under *in vivo* conditions, SA induced state IV respiration as well as oxidative stress in time- and dose-dependent manner, and also inhibited the larval growth. In contrast, JA did not affect the mitochondrial respiration and oxidative stress. SA affected the growth and development of *H. armigera*, in addition to its function as signaling molecules involved in both local defense reactions at feeding sites and the induction of systemic acquired resistance in plants.

Dihydrolipoamide dehydrogenase (DHLDH) was isolated from the mitochondria of *Helicoverpa armigera*. The flavoenzyme was purified 17.98-fold to homogeneity with an overall yield of 10.53% by employing ammonium sulfate precipitation, hydroxylapatite chromatography and CM-Sephadex chromatography. The purified enzyme exhibited the specific activity of 18.7 U/mg and was characterized as a dimer with a subunit mass of 66 kDa. The enzyme showed specificity for NADH and lipoamide, as substrates, with K_m of 0.083 mM and 0.4 mM, respectively. The reduction reaction of lipoamide by the enzyme could be explained by ping-pong mechanism. The Spectra of DHLDH showed the maximum absorbance at 420 nm, 455 nm and 475 nm. The enzyme activity was strongly inhibited by mercurial and arsenical compounds. The N-terminal sequence of Ha-DHLDH showed homology with those of mammalian and arthropod DHLDH. Since *H. armigera* has developed high level of resistance to commonly used insecticides, biochemical properties of the metabolic enzymes such as DHLDH, could be helpful to develop insecticidal molecules for the control of *H. armigera*, with a different mode of action.

Mitochondrial P-glycoprotein (Pgp) was detected in the mitochondrial fraction from larvae of this pest using C219 antibodies, and its role in transport of a rhodamine derivative (TMR) was demonstrated using spectrofluorometric kinetic assay. The TMR accumulated in mitochondria in the absence of ATP, and effluxed out in the presence of ATP. The process of efflux was inhibited in the presence of sodium vandate, an inhibitor of Pgp, in insecticide-resistant larvae of *H. armigera*. At 100 µM concentration, methylparathion inhibited 77.78% and 20.68% of the oxygen consumed by the mitochondria isolated from insecticide-susceptible and -resistant larvae, respectively, compared to the untreated control mitochondria. Insecticides induced a significant release of cytochrome c in the mitochondria isolated from insecticide-susceptible larvae, whereas the release of cytochrome c was inhibited in the mitochondria from insecticide-resistant larvae. Membrane potential decreased in a dose-dependent manner in presence of higher concentration of insecticides (> 50 µM) in mitochondria isolated from insecticide-resistant larvae. Pgp detected in the mitochondria may protect mitochondrial DNA and the components of the electron transport chain from damage due to insecticides in insecticide-resistant *H. armigera* larvae.

Storage proteins, such as arylophorin, are synthesized from the fat body of a wide range of lepidopteran and dipteran larvae and also in other insect orders. Storage proteins of *H. armigera* was obtained by ammonium sulphate fractionation and anion-exchange chromatography. Subunit molecular weight of purified storage protein was estimated to be 66 kDa . The native molecular weight of the protein was determined to be 400 kDa. Amino acid composition showed that the protein was characterized by the presence of aromatic amino acids. Arylphorin purified was identified by data obtained by LCMS sequence. The

deduced amino acid sequence was found to bore homology with the arylphorins of *Spodoptera litura, Manduca sexta, Hyalophora cecropia,* and *Antheraea pernyi.*

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Publications

LIST OF PUBLICATIONS FROM THESIS

- Akbar SMD, Jayalakshmi SK, Sharma HC, Sreeramulu K, 2011, Characterization of dihydrolipoamide dehydrogenase from the mitochondria of *Helicoverpa armigera*, a pest resistant to insecticides, *Entomological Research*, 41**:** 221–228.
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