Chapter 2

Screening of inducible antibacterial proteins by SDS-PAGE
2.1 INTRODUCTION

Insects are ranked as one of the most successful animal groups in terms of total bio-mass and geographical distribution. During 400 million years of existence, insects have rarely succumbed to the evolution of microbial resistance against their potent antimicrobial immune defenses (Eleanor, et al., 2008). The study of insect immunity started during 1920s; and great amount of research on insect immunity began in 1980 with the first isolation and purification of induced antibacterial factors. Innate immunity is the initial response of a host in defending against pathogen invasion (Beutler. 2004; Janeway & Medzhitov. 2002).

The key features of an innate immune response are that it is non-specific (broad-spectrum), rapid (often within minutes to hours) and conserved in its pattern of recognition. Antimicrobial peptides (AMP) are a key component of the innate system and present in virtually all life forms (Zasloff. 2002). In particular, gene encoded antimicrobial peptides are now clearly established as key players in both plant and animal defense systems. In the last two decades, a considerable number of peptides, either inducible or constitutive, and with activity against different types of microorganisms, have been found in almost all groups of animals.

Immunity in insects is composed of cellular immunity and humoral immunity, in this present research we focused on humoral immunity. Humoral immunity involves the secretion of soluble factors into the open circulatory system of the insects. Many of these factors are proceeded from the fat body, the insect homolog of the mammalian liver.

AMPs are small peptides with antimicrobial properties. The number and type vary from insect to insect, although some AMPs are conserved among many species (Bulet, et al., 1999). For example the fruit fly genome analysis has predicted at least 30 different AMPs (Adams, et al., 2000). Experiments with transgenic flies demonstrated that expression of AMPs, in an
immune compromised mutant fly is sufficient to protect against some infections (Tzou P, et al., 2002). Most insect AMPs are thought to function by disrupting bacterial membranes (Lemaitre B & Hoffmann J. 2007). In addition to antimicrobial activities there are examples of vertebrate AMPs acting as mitogens, chemoattractants, and microbial associated molecular patterns (Oppenheim & Yang. 2005). The majority of antimicrobial proteins detectable in the haemolymph after wounding are produced by the fat body, even haemocytes also produce AMPs, during infections in the absence of wounding, the barrier epithelia are also reported to produce AMPs (Basset, et al., 2000).

Humoral responses involve the induced synthesis of anti-bacterial proteins of varied molecular weights such as Cecropins (4 kDa), Attacins (12 to 23 kDa), Diptericins (8 kDa) and Defensins (4 kDa). Humoral reactions require several hours for their full expression and involve in induced synthesis of several antibacterial proteins. These proteins are small and strongly basic. Antimicrobial peptides (AMPs) are part of the armament that insects have developed to fight off pathogens. They exhibit a broad spectrum of antibacterial activity against both gram positive and gram negative bacteria by adapting alpha helical structure on interaction with bacterial membranes resulting in the formation of ion channels. Insects also synthesize lysozyme which enzymatically attack bacteria by hydrolyzing their peptidoglycan or lipopolysaccharide cell walls.

Various types of antimicrobial proteins have been reported in lepidopteran insects and also five major groups of antimicrobial proteins cecropins, insects defensins, attacin like protein, prolin-rich peptides and lysozymes have been isolated in dipteran insects. Most of the novel antibacterial proteins were isolated from lepidopteron haemolymph.
The volume of the hemolymph may be substantial (20–40% of body weight) in soft-bodied larvae (lepidopterons), which use the body fluid as a hydrostatic skeleton, but is less than 20% of body weight in most nymphs and adults. Hemolymph is a watery fluid containing ions, molecules, and cells. It is often clear and colorless but may be variously pigmented yellow, green, or blue, or rarely, in the immature stages of a few aquatic and endoparasitic flies, red owing to the presence of hemoglobin.


It is generally believed that the major function of the blue biliproteins is to provide camouflage for plant-feeding insects (Eleanor R, *et al.*, 2008), but its physiological role in the hemolymph is still unclear. All chemical exchanges between insect tissues are mediated via the hemolymph – hormones are transported, nutrients are distributed from the gut, and wastes are removed to the excretory organs. However, insect haemolymph only rarely contains respiratory pigments and hence has a very low oxygen-carrying capacity. Local changes in haemolymph pressure are important in ventilation of the tracheal system, in thermoregulation, and at molting to aid splitting of the old and expansion of the new cuticle.
Haemolymph provides various kinds of protection and defense from (i) physical injury; (ii) the entry of disease organisms, parasites, or other foreign substances; and sometimes (iii) the actions of predators. In some insects the haemolymph contains distasteful chemicals, which are deterrent to predators. Injury to the integument elicits a wound-healing process that involves hemocytes and plasma coagulation. A haemolymph clot is formed to seal the wound and reduce further haemolymph loss and bacterial entry. If disease organisms or particles enter an insect’s body, then immune responses are invoked. These include the cellular defense mechanisms of phagocytosis, encapsulation, and nodule formation mediated by the hemocytes, as well as the actions of humoral factors such as enzymes or other proteins (e.g. lysozymes, prophenoloxidase, lectins, and peptides).

The immune system of insects bears little resemblance to the complex immunoglobulin-based vertebrate system. However, insects sub lethally infected with bacteria can rapidly develop greatly increased resistance to subsequent infection. Hemocytes are involved in phagocytosing bacteria but, in addition, immune proteins with antibacterial activity appear in the haemolymph after a primary infection. For example, lytic peptides called cecropins, which disrupt the cell membranes of bacteria and other pathogens, have been isolated from certain moths. Furthermore, some neuropeptides may participate in cell-mediated immune responses by exchanging signals between the neuroendocrine system and the immune system, as well as influencing the behavior of cells involved in immune reactions. The insect immune system is much more complicated than once thought.

Insect immunity is well studied in dipterans for instance in Drosophila, mosquito. Only limited information is available in a few lepidopteran species that include the domesticated silkworm *Bombyx mori*, *Hyalophora cecropia* and *Manduca sexta* (tobacco hornworm). Insect
AMPs (antimicrobial proteins) are typically cationic and often made of less than 100 amino acid residues. Although their structures are diverse, most of the AMPs are assigned to a limited number of families. The most common structures are represented by peptides assuming a \( \alpha \)-helical conformation in organic solutions or disulfide-stabilized \( \beta \)-sheets with or without \( \alpha \)-helical domains present. The diverse activity spectrum of these peptides may indicate different modes of action.

Electrophoresis is the migration of charged molecules in response to an electric field. Their rate of migration depends on the strength of the field; on the net charge, size and shape of the molecules, and also on the ionic strength, viscosity, and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid, and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique. There are a variety of electrophoretic techniques, which yield different information and have different uses. Generally, the samples are run in a support matrix, the most commonly used being agarose and polyacrylamide. These are porous gels, and under appropriate conditions, they provide a means of separating molecules by size.

Two commonly used techniques in biochemistry are sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF). SDS-PAGE separates proteins according to molecular weight and IEF separates according to isoelectric point. This chapter exercise will introduces the SDS-PAGE screening of silkworm haemolymph proteins. This technique was introduced by Shapiro et al in 1967. The method provides an easy way to estimate the number of polypeptides in a sample and thus assess the complexity of the sample or the purity of a preparation. SDS-PAGE is particularly useful for monitoring the fractions obtained during chromatographic or other purification procedures.
Denaturation using SDS confers a constant mass-to-charge ratio to all analytes. Smaller molecules migrate faster than large molecules through a gel that acts as a molecular sieve. The primary uses of electrophoresis include determination of both purity of a protein sample and its MW (molecular weight). It is not possible to calculate the physical properties of proteins from mobility data; scientists have taken to denaturing SDS-PAGE in order to estimate molecular weights of proteins. Sample treatment for SDS-PAGE breaks all inner and intra molecular bonds, both covalent and non-covalent, and leaves the polypeptide subunits of proteins in forms that can be separated on the basis of their molecular weights.

SDS solubilizes most proteins, so SDS-PAGE is applicable to a wide range of sample types. Silkworm haemolymph proteins can be difficult to characterize, because of complexity of proteins Haemolymph proteins with a molecular weight (MW) equal to or higher than albumin (69 kDa) are considered to be medium high MW, while those smaller than albumin are classified as low MW proteins. The analysis of haemolymph proteins in silkworm can be useful in the immunological study. Complete genome sequences of silkworm are completed (Mita K, et al., 2004; Wang J, et al., 2005).

Genome and proteome studies on Drosophila have yielded a lot of information for basic studies; however, Drosophila evolutionally belongs to different order than the silkworm. Hideyuki et al 2006 constructed a silkworm proteome database (SPD) to gain an understanding of insect metamorphosis and to apply the information obtained to the future production of useful substances using silkworms. After genome maps of the silkworm were published in 2004 and 2008 (Xia Q, et al., 2004), proteomic technology was vastly applied in silkworm research. Here we present haemolymph proteomic analysis of the immune-challenged larvae of silkworm. This
study followed previous attempts to isolate and characterize new antimicrobial compounds from Silkworm larvae.

In this study SDS-PAGE was used for screening of different silkworm races to identify a novel expressed antibacterial protein.

2.2 MATERIALS AND METHODS

2.2.1 Insects

*Bombyx mori* layings were procured from Central Sericultural Germplasm Resource Center (CSGRC) Hosur, Tamilnadu. **Bivoltine** (*NB7, NB18, NB4D2, PAM 101, PAM 111*) and **Multivoltine** (*Pure mysore, Mysore princes, Kolar gold, Kollegal jawan, Nistari*) races were used for the present study. *Bombyx mori* larvae were reared in room temperature at 27°C on fresh S36 variety mulberry leaves and maintained as per the standard rearing method [Krisnaswami 1973].

2.2.2 Bacterial inoculum

Bacteria were isolated from silkworms infected with bacterial diseases in Dharwad and surrounding villages. Cadavers were collected for bacterial isolation and culturing. Septicemia casual pathogen (*S. aureus*) was incubated in nutrient broth (NB) and log phase *S. aureus*. Bacteria were collected and washed twice with insect physiological saline solution (Table 2.1) (Hiroyuki Ai, *et al.*, 1995).
Table 2.1 Composition of Insect physiological saline (pH 6.5)

<table>
<thead>
<tr>
<th>Name of the chemical</th>
<th>Concentration or Mole (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>28mM</td>
</tr>
<tr>
<td>KCl</td>
<td>16mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>9mM</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.5mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>18mM</td>
</tr>
<tr>
<td>Sucrose</td>
<td>175mM</td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
</tr>
</tbody>
</table>

2.2.3 Immunization

The fifth instars 2 day old larvae were injected with $1 \times 10^5 / 20 \mu l$ of bacterial suspension using Hamilton syringe. Total 50 larvae were immunized and 50 controlled larvae were also reared by injecting insect physiological saline solution at the same condition, the batches were mentioned in triplicates.

2.2.4 Collection of haemolymph

Diseased larvae with bacterial septicemia symptoms were chilled on ice for at least 20 min. The larval caudal horn or pro leg in thoracic region was clipped to release the haemolymph into individual microcentrifuge tubes with few crystals of PTU (phenylthiourea). Haemolymph was collected from the immunized as well as control silkworms at different time intervals post infection (0hr, 24hr, 48hr, 72hr and 96hr) Hemocytes were separated from the haemolymph by centrifugation at $8000 \times g$ for 6-12 min at 4°C depending on developmental stage and stored at -20 °C until further use.

2.2.5 Protein determination

Collected haemolymph from different races immunized as well as from control batches was determined by using Bradford method (Bradford, MM 1976). Briefly standard BSA 1mg per 1ml was diluted in milli Q water with final concentration was 0.1mg/ml. 11 clean test tubes
were taken and BSA sample in different concentrations (0, 10, 20, 40, 60, 80, 100mg), final volume was 100μl filled with milli Q water. 5ml of Bradford reagent was added to each tube and mixed. Immunized and controlled haemolymph sample also prepared accordingly. Tubes were kept in dark conditions for 30 min. The absorbance of each sample was measured at 595 nm using UV-visible spectrophotometer.

2.2.6 Sample preparation for SDS-PAGE

After determination the protein content the haemolymph samples were prepared for SDS-PAGE. 20μl of haemolymph samples were diluted with 80μl of milli Q water and denatured with 100μl of sample buffer (Table 2.2) and boiled to 100°C for 5 min. The prepared samples were loaded onto the SDS gels.

Table 2.2 Composition of Sample buffer for SDS-PAGE

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris-Cl pH 6.8</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>glycerol</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>1% bromophenol blue</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Milli Q water</td>
<td>6.1ml</td>
</tr>
</tbody>
</table>

2.2.7 Standardization of SDS-PAGE protocol for analysis of silkworm haemolymph proteins

Four different gel percentages of 6%, 10%, 15% and 20% were used in the experiment. Different gel percentage was used to analyze various molecular weight proteins present in silkworm haemolymph. Electrophoretic separation was carried out using Bio-rad protean tetra cell at 150 V, 30 mA for 45 min. After electrophoresis gel were stained for 1 hour in 0.2% Coomassie brilliant blue R-250 in 25%(v/v) Methanol and 15% (v/v) Acetic acid, followed by
destaining with 25% (v/v) Methanol and 15% (v/v) Acetic acid, and analyzed in Vilber Lourmat gel documentation system to determine the protein molecular weights.

2.2.8 Screening of silkworm haemolymph proteins

The collected haemolymph sample was analyzed by using 15% SDS-PAGE. The electrophoresis was performed as described earlier. The molecular weight marker proteins (phosphorylase b, 97,400 Da; BSA, 66,200 Da; ovalbumin, 45,000 Da; carbonic anhydrase, 31,000 Da; STI, 21,000 Da and lysozyme, 14,300 Da) were purchased from SRL Laboratories.

2.3 RESULTS

2.3.1 Protein concentration in silkworm haemolymph

The total amount of proteins of both control and immune haemolymph was determined by Bradford method is presented in (table 2.3).

Table 2.3 Protein concentration in haemolymph of bivoltine and multivoltine silkworms.

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Silkworm races</th>
<th>Protein quantity in 5μl of haemolymph</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Bivoltine</td>
<td>a. NB4D2</td>
<td>126μg</td>
</tr>
<tr>
<td></td>
<td>b. NB7</td>
<td>130 μg</td>
</tr>
<tr>
<td></td>
<td>c. NB18</td>
<td>120 μg</td>
</tr>
<tr>
<td></td>
<td>d. PAM 101</td>
<td>117 μg</td>
</tr>
<tr>
<td></td>
<td>e. PAM 111</td>
<td>117 μg</td>
</tr>
<tr>
<td>2. Multivoltine</td>
<td>f. Kolar gold</td>
<td>120 μg</td>
</tr>
<tr>
<td></td>
<td>g. Kollegal jawan</td>
<td>110 μg</td>
</tr>
<tr>
<td></td>
<td>h. Pure mysore</td>
<td>118 μg</td>
</tr>
<tr>
<td></td>
<td>i. Mysore princess</td>
<td>124 μg</td>
</tr>
<tr>
<td></td>
<td>j. Nistari</td>
<td>108 μg</td>
</tr>
</tbody>
</table>

2.3.2 Analysis of silkworm haemolymph proteins by SDS-PAGE

Effective separation of silkworm haemolymph was achieved following appropriate haemolymph dilution and using 15% SDS-PAGE gels. Ten silkworm races, five bivoltine and five multivoltine were analyzed. Our observations reveal that 6% and 10% gels (Fig 2.1 & 2.2)
Analysis of Silkworm haemolymph proteins by using
Varied gel percentages

Fig 2.1: Silkworm haemolymph analysis using 6% SDS-PAGE

Fig 2.2: Silkworm haemolymph analysis using 10% SDS-PAGE

Fig 2.3: Silkworm haemolymph analysis using 15% SDS-PAGE

Fig 2.4: Silkworm haemolymph analysis using 20% SDS-PAGE

Comassie Brilliant blue stained protein bands of silkworm haemolymph proteins separated by SDS-PAGE following 20µl loading of protein onto polyacrylamide gel.
are suitable for analyzing high molecular weight proteins (> 90 KDa), 15% gel (Fig 2.3) is effective for separation of medium range molecular weight proteins (in the range of 15 KDa to 90 KDa) and 20% gel (Fig 2.4) is effective for low molecular weight protein separation. Hence the technique for analysis of silkworm haemolymph proteins was standardized.

2.3.3 Screening of silkworm haemolymph proteins

After electrophoretic separation of proteins the gels were analyzed using Vilber Lourmat gel documentation system for determination of protein molecular weight and protein density. It was observed that bivoltine races were more responsive to immunization as noticed in the protein profile changes of healthy and immune haemolymph proteins. Amongst the five bivoltine races studied, only one new type of inducible protein was expressed in bivoltine races NB7 & NB18. The new protein expressed in NB7 race on fifth instar 4th day and NB18 on fifth instar 2nd day with molecular weight of protein was 31.5 kDa. In remaining races of bivoltine and multivoltine did not show expression of inducible proteins.

SDS-PAGE analysis of fifth instar first day control haemolymph NB4D2 silkworm shows 17 proteins. Prominently expressed proteins are of high molecular weight in the range of 110 to 5 kDa. Amongst these proteins 66 & 30 kDa are prominently expressed (Fig 2.5). While after immunization day 1 to 6 haemolymph analysis showed proteins separated with molecular weight of 100 to 110 kDa. There was no significant difference in haemolymph profiles from day 1 to 6.

In NB7 silkworm haemolymph by SDS-PAGE analysis one new protein of 31.5 kDa was expressed on 4th day of immunization. In our observation 30 and 45 kDa ribosomal proteins showed low level expression on 2nd and 6th day 66 kDa protein was expressed predominantly.
SDS-PAGE Analysis of Bivoltine race Silkworms haemolymph Proteins

Fig 2.5: SDS-PAGE (15% gel) of immune challenged NB4D2 silkworm haemolymph. M: Medium range Protein marker, C: control haemolymph, 1-6: immune haemolymph (Day 1 to 6 post infection)

Fig 2.6: SDS-PAGE (15% gel) of immune challenged NB7 silkworm haemolymph. M: Medium range Protein marker, C: control haemolymph, 1-6: immune haemolymph (Day 1 to 6 post infection)
compared to the remaining days. 30 kDa proteins were abundantly expressed on day 2, 3, 5, 6 post infection compared to control haemolymph (Fig 2.6).

In NB18 silkworm race haemolymph profile showed expression of two new proteins on day 1 of immunization. Total 30 protein bands were identified on day of immunization. The expressed ribosomal proteins molecular weights were 23.9 kDa and 31.5 kDa respectively. 45 kDa protein band showed low level expression in day 2 compared to remaining days. 66 and 30 kDa proteins were showed high level expression on day of 3, 4, 5, 6. (Fig 2.7)

PAM 101 and PAM 111 bivoltine race silkworm haemolymph profiles were similar in control and immunized haemolymph protein separation. Total 18 protein bands were observed in these races. 101 kDa zinc finger protein showed prominent separation. (Fig 2.8 & 2.9).

Similarly multivoltine silkworm haemolymph proteins were analyzed, first day control haemolymph of Kolar gold showed 16 protein bands, we observed 30 kDa protein showed increased expression up to day 3 post immunization and showed decreased expression after 4th day to 7th day (Fig 2.10).

In Kollegal jawan race haemolymph showed 15 different molecular weight protein bands. 30 and 66 kDa proteins showed slow increased expression up to 5 day and decreased on 6th & 7th day post infection (Fig 2.11).

In Pure mysore race control haemolymph showed 17 different molecular weight protein bands. 30 and 66 kDa proteins were more prominently expressed in this race compared to other races protein profiles, there was no difference in protein profile of control and immunized haemolymph protein samples (Fig 2.12).

Haemolymph protein profile of Mysore princess silkworm race showed 22 different molecular weight proteins in control haemolymph. In immunized silkworms from 2nd day to 7th
Fig 2.7: SDS-PAGE (15% gel) of immune challenged NB18 silkworm haemolymph. M: Medium range Protein marker, C: control haemolymph, 1-6: immune haemolymph (Day 1 to 6 post infection)

Fig 2.8: SDS-PAGE (15% gel) of immune challenged PAM101 silkworm haemolymph. M: Medium range Protein marker, C: control haemolymph, 1-6: immune haemolymph (Day 1 to 6 post infection)

Fig 2.9: SDS-PAGE (15% gel) of immune challenged PAM111 silkworm haemolymph. M: Medium range Protein marker, C: control haemolymph, 1-6: immune haemolymph (Day 1 to 6 post infection)
SDS-PAGE analysis of Multivoltine race silkworms haemolymph proteins

Fig 2.10 SDS-PAGE (15% gel) of immune challenged Kolar gold silkworm haemolymph. M: Medium range Protein marker, C: control haemolymph, 1-7: immune haemolymph (Day 1 to 7 post infection)

Fig 2.11: SDS-PAGE (15% gel) of immune challenged Kollegal jawan silkworm haemolymph. M: Medium range Protein marker, C: control haemolymph, 1-7: immune haemolymph (Day 1 to 7 post infection)
Fig 2.12: SDS-PAGE (15% gel) of immune challenged **Pure Mysore** silkworm haemolymph.  
M: Medium range Protein marker, C: control haemolymph, 1-7: immune haemolymph (Day 1 to 7 post infection)

Fig 2.13: SDS-PAGE (15% gel) of immune challenged **Mysore princess** silkworm haemolymph.  
M: Medium range Protein marker, C: control haemolymph, 1-7: immune haemolymph (Day 1 to 7 post infection)

Fig 2.14: SDS-PAGE (15% gel) of immune challenged **Nistari** silkworm haemolymph.  
M: Medium range Protein marker, C: control haemolymph, 1-7: immune haemolymph (Day 1 to 7 post infection)
day of post immunization 30 kDa protein was expressed abundantly compared to control. On day 2, 4 and 7th day 66 kDa protein was highly expressed. Molecular weight of 69.4 kDa protein was not expressed on 1st and 2nd day of after immunization (Fig 2.13).

SDS-PAGE analysis of Nistari silkworm race protein profile of both control and immunized haemolymph, revealed 16 different protein bands and showed identical protein profile both in control and immunized haemolymph protein samples. Proteins of 30 and 66 kDa expression were very high (Fig 2.14).

2.4 DISCUSSION

The silkworm, B. mori, is a domestic insect that has been continuously selected to improve silk production in sericulture for thousands of years. Since the silkworm can be easily reared in a laboratory, B. mori is also a good experimental insect and is now considered to be one of the most thoroughly studied lepidopteron insects in the field of physiology, biochemistry, pathology, and genetics (Kaito C, et al., 2002).

In silkworm, two differentiated fat body (FB) tissues are known by their embryonic origin (Anderson D T. 1972). FB tissue, which is situated at the epidermis or integument region, is called peripheral (PP) or larval FB tissue and it is also known as synthesis site. Most of the major haemolymph proteins are synthesized by PPFB tissue and are secreted into haemolymph. Haemolymph is very important for the metamorphosis of other insects like silkworm.

Proteomic analysis of haemolymph was attempted in fruit flies, mosquito and cattle tick. Haemolymph transports several metabolites. Although the contents of haemolymph and the activities of enzymes such as amino transferase, phosphatase, carbohydrate, and lipid metabolism and the molecular mechanism during immune mechanism is still not well-understood. Vitellogenin one of the main storage proteins in yolk, is considered to be synthesized in fat
bodies and to travel to the eggs in the ovaries via haemolymph. Mine et al. 1983 have reported that the 30 kDa proteins are a specific type of plasma protein and one of the so-called “storage proteins” in the silkworm haemolymph being synthesized in FB of the feeding larvae and released into the haemolymph. During the larval-pupal transformation, they are transported from the silkworm haemolymph to FB cells and stored as protein granules.

Insects prepare for synthetic demands of molting, metamorphosis and reproduction by accumulating the proteins in their haemolymph during the active feeding period (Anishree V, et al., 2005; Pan M L, et al., 2001; Hunt J H, et al., 2003; Telfer W H, et al., 2003; Somasundaram P, et al., 2004). In insects, juvenile hormone regulates the biosynthesis of major plasma proteins (Chinzei Y, et al., 1992). Some investigators have reported that normal haemolymph of various insects can kill some kinds of bacteria (Briggs. 1958; Kawarabata. 1971). It is also well known that a transient bactericidal activity can be induced in the haemolymph of many insect larvae or pupae by vaccination with homologous or unrelated bacteria, endotoxin, and Indian ink, although little or no bactericidal activity was observed in the cell-free haemolymph of nonvaccinated larvae.

Pilot studies on insect innate immunity were performed using the fruit fly, Drosophila melanogaster, as a model organism (Lemaitre B & Hoffmann J. 2007). Further studies on the immune systems of other insect species, such as Lepidoptera, contributed to our understanding of the processes and uncovered the differences in their host defense strategies.

Exploration of immunity in invertebrates is dominated by mechanistic studies of model organisms, with less attention to natural populations (Little, et al., 2005). Here we present a proteomic analysis of the immune-challenged larvae of silkworm. This study followed previous attempts to isolate and characterize new antimicrobial compounds from silkworm larvae. Insects
can produce a variety of AMPs in response to microbial infection or injuries, and most of these peptides are synthesized by the fat body, which is a major insect tissue involved in the immune response. AMPs are secreted into the haemolymph and are responsible for the growth arrest of the microbe at an early stage in the immune response (Eleanor R, et al., 2008). More than 200 AMPs have been identified and characterized in insects (Boman. 1995; Bulet, et al., 1999)

In our present study we focused on standardizing SDS-PAGE protocol for screening of inducible antibacterial proteins in different silkworm races. Antibacterial proteins are low molecular weight proteins. 15% gels were ideal for good resolution for low molecular weight proteins. In silkworm haemolymph approximately eleven types of different proteins were reported (Hideyuki K, et al., 2009). These proteins are functionally and structurally different types. In silkworm twelve types of proteins such as Hypothetical proteins, Zinc finger proteins, DCP2, box A, NUDIX hydrolase, Ribosomal proteins, RNA binding protein, Serpins proteinase inhibitors, Esterase, Ubiquitin like protein, Class I peptide chain release factor, Lipoproteins, Juvenile hormone binding proteins were identified in haemolymph.

Immune peptides have been intensely studied in only a few insect species, including Hyalophora cecropia, Bombyx mori, Drosophila melanogaster, Calliphora vicina, Anopheles gambiae, and Bombus pascuorum (Rees J A, et al., 1997; Chernysh S I, et al., 2000; Vizioli J, et al., 2001; Hultmark D. 2003; Wang Y, et al., 2004). In Drosophila, the five distinct antibacterial peptides represent one of each of the major antibacterial peptide families (Bulet 1999). These peptides are cecropin, defensin, drosocin, diptericin and attacin. Two additional antifungal peptides, drosomycin and metchnikowin, are also secreted.

In silkworm total 8 antibacterial proteins were isolated induced by different bacterial pathogens. Silkworm fat bodies are activated when the injected with non living organisms like
lipopolysaccharide (LPS) and peptidoglycan (PG) (Yongqiang W, et al., 2004). Cecropins can be selectively induced by injecting live or attempted bacterial cell wall components such as PG and LPG. For the induction of cecropin synthesis, PG fragments of some definite structure are required and are suggested to act as a single molecule in *Bombyx mori*. Recently two novel antibacterial proteins belonging to the cecropin family have been identified from *B. mori* (Kim SH, et al., 1998; Axen A, et al., 1997).

Kiyoko Taniai *et al.*, worked on silkworm *B. mori* gene expression and reported the presence of three different antibacterial protein genes *Bm* cecropin B, *Bm* attacin and Labocin. These three antibacterial proteins are simultaneously related in its structural and functional components of the bacterial cell wall.

Isao Morishima *et al.* reported the induction and characterization of antibacterial proteins in hemolymph of silkworm *B. mori*. The antibacterial proteins are only effective as agents. The induction agents were live and heat killed bacteria of both gram positive and negative. But not effective against biotic and abiotic agents like yeast cells, fungal spores, saline, and glass beads *etc*. Actually, antibacterial proteins are effective only on lipopolysaccharids and peptidoglycon compounds, which are components of in bacterial cell wall.

Cecropin B genes and attacin cDNA are detected in the posterior portion of the silkworm body (Tania K., *et al.*, 1997). They injected the bacteria at the posterior portion of the silkworm body, and detected mRNA by northern blot hybridization. In this, the clear cecropin B probe gave positive signal with RNA sample. When saline, *E. coli* was injected it resulted in positive signals in *E. coli* injected portion, but did not give any signal in saline sample injected portion.

Kiyoka Taniai *et al.* used the LPS (Lipopolysaccharide), KDO (2-Keto-3-deoxyxylonate), Lipid A and PG (Phospho glyceraldehyde) these are both gram positive and negative bacterial
cell wall compounds. These compounds were injected into the silkworm body and then induced into hemolymph. In the hemolymph upstream these compounds exhibit the antibacterial proteins. The sequence of moricin, from *B. mori* apparently does not resemble that of any other antibacterial peptide (Hara S, *et al.*, 1995). Some of the silkworm expressed functional proteins were very small, for example bombyxin (78 amino acid residues) (Kondo, *et al.*, 1996), ubiquitin (76 amino acid residues), *B. mori* antibacterial peptide cecropin B (63 amino acid residues; Taniai *et al.*, 1992), a probable cell surface adhesion molecule (30 amino acid residues; Cuadros-Orellana *et al.*, 2007), and the antibacterial peptide astacin (24 amino acid residues; Jiravanichpaisal, *et al.*, 2007). The smallest antibacterial peptide expressed in *B. mori* consists of only 30 residues (Boman, 1995).

Ganesh *et al.*, purified one novel AMPs from silkworm midgut called P252, MW is 252 KDa. It has strong affinity to Cry1A from *B. thuringienesis* bacteria and also shows red fluorescent characters (Ganesh, *et al.*, 2008).

### 2.5 CONCLUSION

Silkworms are classified as univoltine, bivoltine and multivoltines based on their voltinism. Voltinisms also reflect on the sturdiness of these insects to withstand various abnormal situations. Multivoltine silkworm races are sturdy due to their genetic structure. While the univoltine and bivoltine silkworm races have almost double productivity per unit in comparison to multivoltine, univoltine and bivoltines in turn are highly susceptible to diseases or in other words immune compromised and hence are equipped with very strong humoral immunity in the form of inducible antibacterial proteins. Hence we observed the expression of antibacterial proteins in the form of 31.5 kDa in NB_7 and NB_15 bivoltine silkworm race. These inducible antibacterial proteins play a vital role in safeguarding the health of the insects and reflect their
ability to survive against odds of nature. The present investigation has yielded two novel probable antibacterial proteins which will be further analyzed and discussed in detailed in Chapter 4 and 5.