"Research is to see what everybody else has seen, and to think what nobody else has thought"

Albert Szent-Gyorgyi

Chapter 4

Identification, Expression and Characterization of Antimicrobial Peptides from Hyblaea puera C.
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All living organisms undergo a considerable amount of stress in order to survive in contagious environment. This ability to survive is called adaptation which is regulated by immune response. This immune response is highly specific to local environment. In vertebrates, this highly specific immune response is based on the selection of somatically recombined B and T cell receptors while invertebrates and plants lack this type of adaptive immunity. Instead, they survive and diversify under difficult environments with their highly sophisticated adaptive immune response namely innate immunity. Antimicrobial peptides (AMPs) are the key component of the innate immune systems of most multicellular organisms. The expression of AMPs can be constitutive or can be inducible by infectious or inflammatory stimuli, such as proinflammatory cytokines, bacteria or bacterial molecules such as lipopolysaccharides can induce innate immunity.

The antibacterial defense reactions in insect orders like Lepidoptera, Diptera and Hymenoptera are characterized by the synthesis of a battery of potent bactericidal peptides. An increasing number of studies on identification and characterization of AMPs from various taxa is rapidly expanding due to their untapped potential in therapeutic applications (Bowman, 2003). AMPs are exceptionally diverse at sequence level, structurally and functionally even within closely related species. More than 500 antimicrobial peptides have been reported so far from different plants and animals (Wang and Wang, 2004). This has renewed the thoughts on an economic importance of pest species.

4.1.1 INSECT IMMUNE SYSTEM

Insect's immune system is the most primitive when compared to higher vertebrates, but still it efficiently functions ahead during microbial infections.
Innate immunity in the multicellular organisms is the first line of defense mechanism exhibited against the invading pathogen (Hoebe et al., 2004). The immune response in insects is manifested in at least three ways: first a humoral response generates circulating AMPs; second a cellular response that results in phagocytosis or encapsulation of pathogens and third, a phenoloxidase reaction that deposits black melanin around the wound called melanisation (Vilmos and Kurucz, 1998).

4.1.1.1 Humoral Responses

The inducible AMPs are the most important effectors of the humoral responses. Hemolymph clotting is also a humoral response. In insects, two types of clotting mechanisms have been reported. One is based on Ca$^{2+}$ dependent transglutaminase that is released from the hemocytes which catalyse the polymerization of lipophorin and vitellogenin like proteins (Barwig, 1985; Doolittle and Riley, 1990). The second type of coagulation is a three step serine protease cascade that activates the lipopolysaccharide or the (1-3)-β-D-glucan which results in coagulation. In insects hemolymph clotting has structural homology with higher mammalian blood clotting process. This may also suggest a common ancestral origin for the serine protease cascades which play dual role in the insect defense system; it acts as an intermediate component and the proclotting enzymes along with defensin-like domains in hemolymph clotting (Muta and Iwanaga, 1996). It is also involved in the melanin formation (Nappi et al., 1995).

4.1.1.2 Cellular Responses

The cellular response involves phagocytosis, nodule formation and encapsulation. Morphologically hemocytes are distinguished into plasmatocytes, lamellocytes, crystal cells, and these are concerned with different functions. Plasmatocytes are involved in phagocytosis activity in clearing intruders. Lamellocytes forms the cellular capsule around the intruder. Crystal cells are
engaged in phenol oxidase reaction where black pigment melanin gets released through cell rupture and encapsulate the intruder (Hultmark, 2003).

The molecular aspect of phagocytosis in insects is not well studied. Granular cells and plasmatocytes are chiefly responsible for phagocytosis (Ehlers et al., 1992). Nodule formation is the result of aggregation of hemocytes around the pathogens. Nodule formation has been reported in Manduca sexta, in the epidermal cells and midgut cells upon wounding or bacterial infection (Kyriakides et al., 1995). Encapsulation is a process where the capsules are formed by overlapping layers of hemocytes around the invaders. Encapsulation is further associated with the melanisation (Nappi et al., 1995; Strand and Pech, 1995).

4.1.2 ANTIMICROBIAL PEPTIDES

The AMPs are generally short, ranging from 15 to 45 amino acids in length, cationic and amphipathic. The mature peptides are often cleaved off from a larger protein containing a signal peptide and a propiece (Bowman, 2003). The mature peptides are active against a wide range of microbial lipid layers. Being positively charged, they bind to anionic microbial lipid bilayer and disrupt them non-catalytically. Drosophila is a renowned model organism whose complete genome information paved way to identify at least 34 antimicrobial peptides belonging to eight major families. Within these eight families each family has their own spectrum of action. Most of the antimicrobial peptides so far discovered from various insects are inducible, have relatively low molecular weight and are positively charged. These peptides differ among themselves either structurally, functionally or differ by post-translation modification or by mode of action. More than 150 antimicrobial peptides were isolated from insects and were grouped mainly into four different families (Yamakawa and Tanaka, 1999). The inducible antimicrobial peptides are important effectors of the humoral response in lower invertebrates (Hetru et al., 1998; Bulet et al., 1999). The evolutionary
relationships between these gene families are not clearly understood. It is suggested that AMP gene ancestors underwent several round of duplication, giving rise to different gene families in the genome. As a result, AMP genes exhibit species-specific expansion which permits the genes involved in the innate immunity to get finely tuned to the multiple pathogens.

AMPs are effective against antibiotic-resistant pathogenic bacteria such as methicillin-resistant S. aureus (MRSA) and P. aeruginosa which suggest their importance in the therapeutics (Yamada and Natori, 1993; Miyanoshita et al., 1996; Hara et al., 1996). Amphipathic α-helical region has been identified as active sites of antibacterial peptides (Ojcius and Young, 1991; Blondelle and Houghten, 1992; Yamada and Natori, 1994). Modifications of these AMPs can lead to greater and broader antibacterial activity than the original peptide (Alvarez-Bravo et al., 1994; Helmerhorst et al., 1997). The C-terminal β-sheet domain of AMPs were found to be an active site and demonstrated to have activity against fungi and G^+ve and G^-ve bacteria (Lee et al., 1998). Designing oligopeptides based on the available amino acid sequences from the insects had effectively demonstrated for its enhanced activity against G^+ve and G^-ve bacteria (Saido-Sakanaka et al., 1999; Ishibashi et al., 1999). Based on this background information of insect immune system and its potential, this part of the study was initiated on altering the notion of a pest species into an economically viable species.
4.2 AIM OF THE STUDY

4.2.1 TO IDENTIFY AND ISOLATE AMPS FROM *H. puera*

Teak defoliator is recognized as a forest pest which prospers in stressful environment invaded by many natural pathogens. This current study intends to convert the notion of the pest into an insect of higher economical importance. Therefore, the first objective of the study was to identify and isolate antimicrobial genes from the immune-challenged *H. puera* larvae.

4.2.2 *IN SILICO* CHARACTERIZATION OF AMPS

After isolation of AMPs the next immediate need was to sequence characterize them. *In silico* method provided a good preliminary understanding of the AMPs based on its sequence data. Therefore, the primary objective was to understand the phylogenetics and structural significance of the AMPs isolated from *H. puera* in relation to other existing insect AMPs.

4.2.3 FUNCTIONAL CHARACTERIZATION OF AMPS

The final objective was to functionally validate the AMPs by cloning them in the appropriate expression system. The recombinant AMPs were purified and assayed for their antimicrobial activity.
4.3.1 MATERIALS

4.3.1.1 CHEMICAL AND BIOCHEMICAL REAGENTS

Most of the chemicals and biochemicals used in the study are already mentioned in the previous chapters. Glycine, Methanol, Glacial acetic acid, Tween-20, Hydrogen peroxidase solution (H₂O₂) was obtained from SRL chemicals, India and Qualigens, India. Triton X-100, Sodium Acetate, LB broth, LB agar, Ampicillin, Dimethyl Sulfoxide (DMSO), 1,4-Dithio-DL-threitol (DTT), Phenylmethanesulfonyl fluoride (PMSF) Acrylamide, Methylene Bis Acrylamide, N,N,N’,N’- Tetramethylethylenediamine (TEMED), Ammonium Per Sulphate (APS), Isopropylthiogalactoside (IPTG), Coomassie Brilliant Blue G250, Diaminobezidine (DAB), Bovine serum albumin (BSA), Protease Inhibitor Cocktail, β-Mercaptoethanol, Xylene Cyanol, Bromophenol Blue and Hybond™-C Extra, TRizol reagent, RNasin (RNase inhibitor) were from Sigma Chemicals, USA and Amersham, USA. BD TALON™ Metal affinity resins were purchased from Becton Dickinson (BD) Biosciences Clontech, USA. Non-charged modified nylon blotting membrane was purchased from Millipore, USA. Sterile discs were obtained from HIMEDIA Laboratories. Pvt. Ltd, India.

BamH I, Nde I, Sac I, Sac II, Spe I and Xho I restriction enzymes and broad range prestained protein marker (6-175 kDa) were acquired from New England Biolabs Inc., USA. M-MLV reverse transcriptase, nuclease free water and T4 DNA Ligase were obtained from Promega, USA. Custom made primers were synthesized from (Sigma Genosys, India). Glasswares and plasticwares were mentioned in the earlier chapter.

4.3.1.2 INSTRUMENTS

Mini PROTEAN® 3 cell, Mini Trans-Blot® cell (Bio-Rad, USA), ECL Semi-dry blotter (Amersham biosciences, USA) was used for SDS-PAGE and western blot. Sonifier 450 (Branson Ultrasonics Corporation, USA) was used to disrupt the bacterial cells.
4.3.1.3 **BACTERIAL STRAINS**

Genotypes of the bacterial strains used in the current study are given below.

- **DH5α**: F, (endA1, recA1, glnV44, thi-1, relA1, gyrA96, deoR, nupG, [Φ80d lacZΔM15 Δ (lacZYA-argF)] U169, hsdR17 (rK− mK−), λ−
- **JM109 (DE3)**: (endA1, recA1, gyrA96, thi, hsdR17 (rK−mK+), relA1, supE44, Δ(lacproAB), [F',traD36, proAB, lacIΔZΔM15], λ(DE3)]
- **BL21 (DE3) pLysS**: F' ompT gal dcm lon hsdS_{B}(r_{B}^- m_{B}^-) λ(DE3) pLysS(cmR) were obtained from **Promega** or **Novagen**, USA.

4.3.1.4 **VECTORS**

The pGEM®-T Easy vector and pGEM®-T vector from **Promega** was used to clone the PCR products. pBluescript II SK+ was obtained from **Stratagene**, USA. The prokaryotic expression vectors pET-32+ and pET-31b+ were obtained from **Novagen**.

4.3.1.5 **ANTIBODIES**

The primary monoclonal Anti-polyHistidine antibody and the secondary Anti-Mouse IgG horseradish peroxidase (HRP) conjugate were obtained from **Sigma**.

4.3.1.6 **SOFTWARES EMPLOYED**

**Primer Premier v 5.00** (**Premier Biosoft International**, USA) software was used to design the custom primers, **SimVector v 2.00** (**Premier Biosoft International**, USA) software was used to draw the vector maps. **Sequence Scanner v 1.0** (**Applied Biosystems**) software was used to analyze the raw sequences. **BioEdit Sequence Alignment Editor v 7.0.5.3** (Hall, 1999) with **Clustal-W v 1.4** multiple alignment software interface (Thompson *et al.*, 1994) was used to align the sequences.
Phylogenetic trees were constructed using MEGA v 3.1 (Kumar et al., 2004). Swiss-Pdb Viewer v 3.7 (http://www.expasy.org/spdbv). Homology models generated were validated using the SWISSMODEL protein-modeling server (http://www.expasy.ch/swissmodel/). PDB format files were visualized using Molsoft ICM v 3.4-8 software. The theoretical isoelectric point (pl) and molecular weight (Mw) were calculated using the online expasy tool http://au.expasy.org/tools/pi_tool.html.

4.3.2 METHODS

4.3.2.1 IMMUNIZATION

The H. puera larval samples were collected from the field and reared to F1 generation. The mature fifth instar larvae were immunized. The immunization of larval samples were carried out by injecting 5 µl of E. coli culture containing approximately $10^6$ cells suspended in 1 X PBS (Phosphate-Buffered Saline) using sterile 2 ml disposable syringe.

4.3.2.2 ISOLATION OF AMP GENES

4.3.2.2.1 RNA ISOLATION

The larval samples were thoroughly washed with diethyl pyrocarbonate (DEPC) treated sterile Milli-Q water. Total RNA was isolated from the whole larva by adding 1 mL of TRIzol reagent into the 1.5 mL eppendorf tube. The larvae samples were homogenized using sterile DEPC treated micro pestle (Tarson). The samples were incubated in room temperature for 5 mins. Then 200 µL of chloroform was added to the TRIzol containing homogenized tissue and inverted vigorously. The mixture was incubated in room temperature for 3 mins. The tubes were spun at 13,000 rpm for 15 mins at 4°C. The upper colorless aqueous phase was transferred to fresh DEPC treated tubes. To the aqueous phase 500 µL of isopropyl alcohol was added and mixed thoroughly by inverting. The total RNA was precipitated by incubating the mixture for 10 mins. The tubes
were spun at 13,000 rpm for 15 mins at 4°C. RNA pellet was washed twice with 500 µL of 75% ethanol by centrifugation at 10,000 rpm for 5 mins at 4°C. The RNA pellet was air-dried in RNAse free laminar hood and dissolved in 30 µL of nuclease free water. The quality of the RNA was visually analysed in a 1.2% agarose gel.

4.3.2.2.2 Quantitation of RNA

The quantity of the total RNA was estimated spectrophotometrically at 260 nm. The concentration of total RNA was calculated using the following equation.

\[
\frac{40 \times \text{Optical density (OD)} \times \text{dilution factor}}{1000}
\]

4.3.2.2.3 Isolation of mRNA

mRNA was separated from total RNA using the PolyATtract® mRNA Isolation System III from (Promega) according to the manufacturers' protocol, as described below.

4.3.2.2.3.1 Annealing the Oligo dT Probe

Total RNA was dissolved in 500 µL of nuclease free water and incubated at 65°C for 10 min. To the denatured total RNA, 3 µL of oligo dT probe and 13 µL of 20 X SSC (both provided in the kit) were added and incubated at room temperature for 20 mins to allow the slow cooling of the mix to room temperature.

4.3.2.2.3.2 Washing of Streptavidin-Paramagnetic Particles

The Streptavidin-paramagnetic particles (SA-PMP) were resuspended by gently flicking in order to disperse it completely. To capture the SA-PMPs, the tube was placed in a magnetic stand, until the SA-PMPs were collected on the side of the tube. The supernatant was carefully removed and SA-
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PMPs were washed thrice with 300 µL of 0.5 X SSC. Washed SA-PMPs were resuspended in 100 µL of 0.5 X SSC.

4.3.2.2.3.3 CAPTURE AND WASHING OF ANNEALED OLIGO (dT) – mRNA HYBRIDS

Add the entire content of the annealing reaction to the tube containing washed SA-PMPs. The mix was incubated at room temperature for 10 mins and the tubes were inverted every 1 - 2 mins. The SA-PMPs were captured using the magnetic stand and the supernatant was carefully removed without disturbing the SA-PMP pellet. The particles were washed four times with 300 µL of 0.1 X SSC by gently flicking the bottom of the tube until all particles were resuspended. After the final wash the supernatant was removed without disturbing the pellet.

4.3.2.2.3.4 ELUTION OF mRNA

The final SA-PMP pellet was resuspended in 30 µL of nuclease free water to elute mRNA. SA-PMPs were captured magnetically and transfer the eluted mRNA to a sterile RNase free microfuge tube. The elution was repeated by adding additional 30 µL of nuclease free water. Final elution was carried out with the pooled 60 µL mRNA solution. Eluted mRNA was quantified spectrophotometrically and stored at -80°C till further use.

4.3.2.2.4 FIRST STRAND SYNTHESIS OF cDNA

The first strand cDNA was synthesized from mRNA by using M-MuLV reverse transcriptase. The reaction mix was prepared for 50 µL volume with 4 µg of mRNA, 1 X M-MuLV RT buffer, 200 U of M-MuLV RT (Promega), 0.5 mM dNTPs mix, 5 U of RNasin (RNase inhibitor) and 2.0 µg oligo dT18 primer. For the first strand synthesis mRNA, oligo dT18 primer and nuclease free water were added to DEPC-treated 0.6 mL eppendorf tube and denatured at 65°C for 10 min and snap cooled in order to remove
secondary structures formed in the mRNA. The denatured samples were spun down and M-MuLV RT, RNasin, dNTPs were added to it. The RT reaction mix was incubated at 42°C for 90 mins in a water bath followed by heat inactivation of the enzyme at 75°C for 15 min on a heating block. The samples were stored at -20°C until further use.

4.3.2.5 GENE SPECIFIC cDNA AMPLIFICATION

4.3.2.5.1 PRIMER DESIGNING

The primers for amplifying gene specific cDNAs were designed based on the sequences of the nearest lepidopteran genetic neighbour of H. puera. Among the available moricin sequences in the GenBank, B. mori was found to be the nearest neighbour. Therefore, the moricin primers were designed based on Furukawa et al., (1999).

Primers for the defensin gene were designed based on the conserved regions found on the fly sequence using Primer premier v 5.00 software. Since there is no known report of lepidopteran defensin, we tried to design primers on Stomoxys calcitrans (AF013146) to amplify the cDNA of H. puera. Primers for cloning the defensin gene in expression vectors were designed by incorporating appropriate restriction sites in the 5' and 3' of the gene sequence. The forward primer was designed to incorporate 18 bases coding for six histidines in the N-terminal of defensin peptide. The incorporated histidines play an important role in the purification of recombinant proteins. The primer sequences are mentioned in the table 4.1.

4.3.2.5.2 AMPLIFICATION CONDITIONS OF AMP GENES

Moricin gene was amplified using the cDNA template of the immunized larval samples. The primer morfor was used as the common forward primer to distinguish the gene transcripts of moricin isoforms that was present in the immunized larva. PCR was performed in a total volume of 30 µL. Each reaction consisted of 1 µL of cDNA as template from the
immunized larva, 1X *Taq* buffer with 1.5 mM MgCl₂, 1.2 U of *Taq* polymerase (BG), 0.25 mM of dNTPs (Amersham) and 10 pmol of primers (Sigma Genosys) per reaction. *HpMoricin I* was amplified using morfor as forward and morrevl as reverse primer. *HpMoricin II* isoforms was subsequently amplified using morfor with morrevll primer. Amplifications were performed in a Thermocycler (Bio-Rad) and programmed as follows: initial denaturation at 95°C for 3 mins, followed by 35 cycles of cycle denaturation at 94°C for 1 min, annealing at 48°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 3 mins and held at 4°C.

*Defensin* gene was amplified using similar conditions as mentioned above except for thermocycler conditions. *Defensin* gene was amplified using deffor and defrev primers and the cycling conditions are as follows: initial denaturation at 95°C for 3 mins, followed by 35 cycles of cycle denaturation at 94°C for 1 min, annealing at 55°C for 30 secs, extension at 72°C for 1 min, and final extension at 72°C for 3 mins and held at 4°C. For amplification of *defensin* with His tag and restriction site incorporated primers, the annealing temperature was kept at 65°C. Two different construct were produced by means of two sets of primers. For the 1st set 5' His tag with *Nde* I restriction site and 3' with *Xho* I site was incorporated using DefhisNdel and DefrevstopXhol primer pairs with the cloned plasmid of *HpDefensin* gene. C-terminal His tagging was attained by using DefNdel and DefrevXhol primer pairs to amplify the required recombinant insert. DefrevXhol primer was designed with deleted stop codon on the 3' of the *defensin* gene so as to express the C-terminal His tag from the vector sequence as fusion protein.

4.3.2.2.5.3 Visualizing cDNA Fragments

The PCR products were separated in 1.5% Agarose gel in 0.5 X TBE buffer. To the 30 µL PCR product 3 µL of 10 X gel loading dye was added loaded in the agarose gel. A co-migrating size standard was used to
estimate the size of the PCR product. The gel was stained with ethidium bromide (0.5 µg/mL). The electrophoresis was done at constant voltage of 80 volts. The PCR products were visualized in Fluor-S™ multi imager system (Bio-Rad) using Quantity one software module.

4.3.2.3 Cloning of cDNA Fragments

The PCR products were eluted from the gel by using GFX™ PCR gel band purification column (Amersham) as per the manufacturers' protocol. The gel eluted PCR products were quantified and cloned into the pGEM®-T vector system as per the manufacturers' protocol. The colonies were screened initially by blue-white screening and further confirmed by the colony PCR as mentioned earlier.

4.3.2.3.1 Plasmid Isolation

The plasmid isolation was carried out using QIAgen® Miniprep kit according to manufacturers' instructions. The positive colonies were picked and inoculated in 5 mL LB (Luria-Bertani) medium containing 5 µL of ampicillin (100 mg/mL). The inoculated culture was kept in a shaker cum incubator for overnight at 37°C with 200 rpm. The culture was pelleted by centrifugation for 2-3 mins at 12,000 rpm in the 1.5 mL eppendorf tubes. The pellet was then resuspended thoroughly in 250 µL of P1 buffer containing RNase A. To the resuspended cells 250 µL of P2 Buffer was added and the tubes were inverted 4-6 times until the solution became viscous and clear. Neutralization was done by adding 350 µL of N3 buffer to the cell lysate. The tubes were mixed gently by inverting 4-6 times. The tubes were spun for 10 mins at 13,000 rpm in the microcentrifuge. Bacterial cell debris was pelleted in the bottom of the tube and the supernatant was transferred to the QIAprep spin column. The column containing the supernatant was centrifuged for 30-60 secs at maximum speed and the flow-through collected in the collection tube was discarded. Column was
washed with 750 µL of PE buffer by centrifugation for 30-60 secs at maximum speed. The flow-through was discarded, and the column was centrifuged for 1 min to eliminate the residual wash buffer. The columns were placed in the fresh 1.5 mL eppendorf tube and 50 µL of nuclease free water was added. The QIAprep columns were centrifuged for 1 min at maximum speed and the plasmid DNA was eluted in the fresh tube. The plasmid was quantified in 1% agarose gel in 0.5 X TBE buffer.

4.3.2.3.2 Sequence Characterization

The plasmids are sequenced using T7 and SP6 universal primer present in the pGEM®-T Easy Vector. The sequencing was done in ABI 3730 DNA Analyzer (Applied Biosystems, Perkin Elmer) using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems, Perkin Elmer). The detail protocol is mentioned in the earlier chapter. The raw sequence data were analysed using Sequence Scanner Software v 1.0 for Phred quality scores. Base calls of more than 20 Phred scores were considered and lower base calls were rejected. Sequencing was repeated to observe the repeatability of data obtained.

4.3.2.4 In silico Characterization of AMPs

4.3.2.4.1 Phylogenetic Analyses of AMP Genes

Dataset for the phylogenetic analysis was obtained from the GenBank. The phylogenetic study pertains only to the mature peptide at amino acid levels. All the moricin sequences from the database were selected for the phylogenetic study (Table 4.2). Only seven sequences from the database including HpMoricin were obtained for the phylogenetic study of moricin gene. While defensin gene has more sequence records in the GenBank database, around 31 sequences were selected for the analysis. Sequence Scanner v 1.0 software (Applied Biosystems) was
used to analyze the raw sequences for its quality. Only those isoforms whose mature peptide region has amino acid substitution were considered for the phylogenetic analyses (Table 4.3). Alignments of protein sequences were carried out using Clustal W v 1.4 (Thompson et al., 1994) module of BioEdit v 7.0.5.3 software (Hall, 1999). The phylogenetic trees were constructed by MEGA v 3.1 (Kumar et al., 2004) using Poisson correction model. Neighbour joining (NJ) (Saitou and Nei, 1987) and maximum parsimony (MP) trees were constructed with 1000 bootstrap replications.

4.3.2.4.2 Structural Characterization of AMP Genes

The theoretical isoelectric point (pI) and molecular weight (Mw) were calculated using the online expasy tool http://au.expasy.org/tools/pi_tool.html (Laskowski et al., 1993). Homology models were generated by Swiss-PdbViewer v 3.7 while the pdb files were then visualized in Molsoft ICM v 3.4-8 for better graphics. The stereochemical quality of protein structures of the completed model was validated by submitting to SWISSMODEL protein-modeling server (http://www.expasy.ch/swissmodel/). Ramachandran plots were generated for both templates and for the modeled structures using Molsoft ICM v 3.4-8 and PROCHECK online software (Morris et al., 1992).

4.3.2.5 Functional Characterization of AMPS

4.3.2.5.1 Quantitative Analysis of AMP Transcripts

Semi quantitative RT was carried out to measure the expression level of AMP transcripts. Larval samples were immunized and the RNA isolation was carried out from the whole larva at different time intervals to estimate the accumulation of moricin gene transcripts at 0 hr, 2 hrs, 4 hrs, 6 hrs, 12 hrs, 24 hrs and 48 hrs of post immunization. The immunization was done in triplicates i.e. each set consisted of three larvae maintained at 25°C with artificial diet in a sterile environment. Control set of larvae were
injected with 5 µL of 1 X PBS and RNA isolations were carried out exactly at the speculated course of time.

The semi quantitative RT-PCR conditions were similar as mentioned earlier in the chapter, except for the cycles. For moricin transcript, the PCR was carried out for 30 cycles in order to estimate the copy number at lower cycles. The 28SrRNA gene was employed as the house-keeping for normalization and PCR conditions are mentioned in the earlier chapter.

4.3.2.5.2 Sub Cloning HpMoricin and HpDefensin Genes in Expression Vectors

The clones were initially screened by colony PCR and the recombinant plasmids were isolated. The plasmids were sequenced to check the restriction site sequences. The moricin gene was sub cloned from pGEM®-T vector to pBluescript SK vector, since restriction sites in pGEM®-T vector were not compatible with pET-32 expression vector. The frame of the recombinant moricin was analysed and found that pET-32a vector was in the frame. Spe I and Sac II enzymes are used to release the fragment from the pGEM®-T vector. Restriction digestions were carried in eppendorf tubes in a total volume of 30 µL. The recombinant pBluescript plasmid vector DNA (5 µg) was subjected to double digestion with 20 U of each Spe I and Sac II enzyme (NEB), 1 X restriction buffer 2 [50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol (pH 7.9 at 25°C)] and 1 X BSA. This fragment was cloned in pBluescript SK (+). BamH I and Sac I enzyme sites were selected to sub-clone the moricin gene into pET-32 expression vector. Sequential digestion was carried out with the respective buffers. The pET-32a vector was linearized and subsequently, the insert from the pBluescript vector was released using BamH I and Sac I enzymes (NEB). The restriction digestion conditions were followed as mentioned above except the buffers, for BamH I digestion [150 mM NaCl, 10 mM Tris-HCl,10 mM MgCl₂, 1 mM dithiothreitol (pH 7.9 at 25°C)] and for Sac I [10 mM Bis Tris
CHAPTER-4 MATERIALS AND METHODS

Propane-HCl, 10 mM MgCl₂, 1 mM dithiothreitol (pH 7.0 at 25°C)] supplemented with 1 X BSA. The digestions carried out in 37°C overnight in a water bath.

The defensin gene was incorporated with Nde I restriction site at the 5' and Xho I site at the 3' using cloning primers by PCR. The pET-31b+ vector and the PCR products were subjected to double digestion with 20 U of each Nde I and Xho I (NEB) in 30 µL reaction with 1 X restriction buffer 4 [20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate 1 mM dithiothreitol (pH 7.9 at 25°C)]. The restriction digestion was done overnight at 37°C in water bath. The released fragments were separated in 2% agarose gel with 0.5 X TBE buffer. The fragments were purified using GFX™ gel purification column. Eluted bands were quantified and ligation reactions were performed.

T4 DNA ligase (Promega) was used to ligate the purified DNA fragments with the linearized vectors. Ligations were carried out with an excess of insert DNA (vector: insert molar ratio was usually 1:3). The reaction mix contained 50 ng of linearized vector, 150 ng of insert, 1 X ligase buffer and 1 µL of T4 DNA ligase (3 U). The ligation reaction was carried out at 4°C overnight. The 5 µL of ligation mixture was directly used for transformation into DH5α competent E. coli cells.

4.3.2.5.3 SCREENING RECOMBINANT CLONES

4.3.2.5.3.1 COLONY HYBRIDIZATION

The recombinant colonies were streaked identically in duplicates on LB agar plate containing ampicillin (100 mg/mL). The colonies were lifted onto precut non-charged modified nylon blotting membrane (Millipore). The membrane was marked properly for orientation by cutting the corners. The membranes were serially treated with depurination solution (0.25 N HCl) for 5 mins, denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 10 mins and twice with neutralization solution [1.5 M NaCl, 0.5 M Tris-HCl (pH 8)] for 10 mins. After neutralization the membranes were placed on Whatman filter paper.
soaked in 2 X SSC for 5 mins. The DNA from lysed colonies was fixed to the membrane by UV crosslinking. Hybridization of the membranes was carried out at 65°C in pre hybridization buffer. Amplified PCR products were used as probes by radiolabeling with α32P dCTP. Probe radiolabeling and hybridization procedures were carried out as described in the earlier chapter. After autoradiography, positive clones were selected and plasmid isolation was done using QiAgen® Miniprep kit. The recombinant plasmids were further checked for the orientation using the vector specific primer (S.tag) with the gene specific reverse primer (morrevl). The positive clones were sequenced to ensure that the sub-cloning procedures had not altered the open reading frame (ORF) of the gene.

4.3.2.5.4 REMOVAL OF TAGS FROM EXPRESSION VECTORS

Thioredoxin tag (Trx) was released from the pET-32a+Trx+Moricin vector by Nde I restriction enzyme. Restriction digestion reaction was carried out as mentioned above in 30 µL total containing 5 µg of pET-32a+Trx+Moricin recombinant plasmid, 20 U of Nde I enzyme (NEB) and 1 X restriction buffer 4. The reactions were set for overnight at 37°C in water bath. The digested products are separated in 1.2% agarose gel and the pET-32a+Moricin fragment was purified and self ligated.

4.3.2.5.5 TRANSFORMATION IN EXPRESSION STRAINS

The recombinant plasmids were quantified and approximately 50 ng of pET-32a+Trx+Moricin, pET-32a+Moricin, pET-31b+N-6XHis+Defensin and pET-31b+Defensin+C-6XHis were transformed separately into competent JM109 (DE3) and BL21 (DE3) pLysS expression strains of E. coli. Transformants were selected on LB agar plates containing 100 µg/mL ampicillin. The glycerol stocks were made for the recombinants both in non-expression as well as expression strains and stored in -80°C Deep freezer.
4.3.2.5.6 RECOMBINANT PROTEIN EXPRESSION

4.3.2.5.6.1 INDUCTION OF RECOMBINANT PROTEIN

A single colony from the transformed plate was picked and inoculated in 2 mL LB medium with ampicillin for overnight at 37°C with 200 rpm in shaking incubator. From the overnight culture, 500 µL was again inoculated in fresh 5 mL of LB medium containing ampicillin (50 mg/mL). The cultures were allowed to grow till the optical density reached OD₆₀₀. At this point induction was carried out by adding IPTG into the culture. The standardization of IPTG concentrations were done in small scale for the recombinant proteins. The induction was optimized for at 1 mM and 0.5 mM for moricin and defensin genes respectively. The induction temperature was optimized at 37°C for both AMP genes. The induced fractions were collected on hourly basis then analysed by SDS-PAGE using coomassie staining.

4.3.2.5.6.2 MONITORING PROTEIN EXPRESSION PROFILE

The recombinant protein samples were analysed on a SDS-PAGE (Laemmli, 1970) using a Mini PROTEAN® 3 cell PAGE apparatus (BioRad). A 12% resolving gel and a 6% stacking gel was casted for resolving the recombinant protein (Appendix). Samples were diluted in 3 X sample loading buffer (NEB) [187.5 mM Tris-HCl (pH 6.8 at 25°C), 6% (w/v) SDS, 30% glycerol, 0.03% (w/v) bromophenol blue and 125 mM DTT] heat denatured at 95°C for 10 mins and approximately 20 µL was loaded in each well. Broad range pre-stained protein marker (NEB) was simultaneously loaded and electrophoresised to estimate the protein size in the samples. Electrophoresis was carried out initially at 80 volts. When the samples entered the separating gel, voltage was increased to 100 volts for 2 hrs. Tris-glycine buffer [25 mM Tris base, 192 mM glycine and 0.1% SDS (pH 8.3)] was used for separation.

To visualize the protein profile the gels were stained with Coomassie Brilliant Blue R250 [(0.25% w/v) in 40% methanol: 10% glacial acetic acid:
50% distilled water]. Staining was carried out with gentle shaking for 10 to 15 mins. The gels were destained in an aqueous solution of 30% methanol and 10% glacial acetic acid for about 2-3 hours with gentle shaking. The gel pictures were captured and analyzed using Fluor-S™ multi imager system (Bio-Rad) using Quantity one software module.

4.3.2.5.6.3 CONFIRMATION OF EXPRESSION

Western blot analysis was carried out according to the Towbin et al., (1979) to confirm the AMP expressions. The protein samples were separated by SDS-PAGE as mentioned earlier. Western blotting for thioredoxin tag recombinant proteins was carried out using Mini Trans-Blot® cell (Bio-Rad). For the low molecular weight peptides the transfer was done using ECL Semi-dry blotter (Amersham biosciences). Proteins were electroblotted from 12% or 15% SDS-PAGE gel to the nitrocellulose membrane using towbin transfer buffer [50 mM Tris, 380 mM glycine, and 20% methanol]. The stacking gels were removed carefully and the separating gels were transferred to towbin buffer for 15 to 20 mins. The Hybond™-C Extra nitrocellulose membrane (Amersham) was cut exactly to the gel size and soaked in the transfer buffer for 15 mins before transfer. The unit was assembled as per manufacturers' instructions, and the whole unit was kept embedded with ice during the electrophoretic transfer. This was done to prevent the heat generated during transfer. The transfer was carried out at a constant voltage of 80 volts for 100 mins at 12°C. To ensure complete transfer as well as to detect the size of the recombinant fusion protein, the prestained protein marker was loaded along with the samples. In case of pET32a+Trx-Moricin where the thioredoxin tag was removed, the western blot was done using Semi-dry blotter. The blotting was done as per the manufacturers' guidelines at 20 volt for 15 mins. After transfer the nitrocellulose membrane, was washed twice with TBST [(Tris Buffered Saline with Tween) 50 mM Tris HCl, 150 mM NaCl, 0.1 % Tween 20 (v/v) (pH 7.5)].
The membrane was blocked by incubating in TBST containing 5% non-fat milk powder for 1 hr at room temperature to prevent non-specific binding. The Primary antibody was diluted in TBST containing 3% BSA. The required dilutions were done as per the manufacturers’ guidelines. Immunodetection was done using mouse monoclonal anti-polyhistidine antibody peroxidase conjugate (Sigma) diluted 1:3000. The blot was incubated at room temperature for 2 hrs. The membrane was washed thrice with TBST for 10 mins each and incubated with the secondary anti-mouse HRP antibody (Sigma) which was diluted according to manufacture recipe (1: 5000 in TBST containing 3% BSA). The membrane was incubated for an hour followed by three washes with TBST for 10 mins each. The membrane was developed using 10 mL of 50mM Tris (pH-7.6), 6 mg of 3, 3’-diaminobenzidine (DAB) and 10 µL of hydrogen peroxide.

4.3.2.6 Purification of Recombinant Protein

4.3.2.6.1 Large-Scale Isolation and Characterization

An isolated colony was picked and inoculated in a 10 mL LB broth containing ampicillin for overnight shaking with 200 rpm at 37°C. The JM109 (DE3) cell containing pET32a+Trx+Moricin was inoculated into 500 mL of fresh LB medium containing 100 mg/mL ampicillin and incubated till the growth reached logarithmic phase (OD₆₀₀). The culture was incubated for 15 mins at 25°C prior to the induction. IPTG was added to the culture at a final concentration of 0.5 mM and the culture was incubated for 5 hrs at 37°C. Un-induced controls, JM109 (DE3) cells transformed with the vector alone were grown under similar conditions in small scale. The cells were pelleted in a pre-weighed centrifuge bottle at 5000 rpm for 10 mins at 4°C. The bacterial pellet was resuspended in lysis buffer [50 mM Tris (pH 7.5) 150 mM NaCl, 1% Triton X-100, 5 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF and 1 X Protease Inhibitor Cocktail] and frozen at -80°C. Five mL of lysis buffer was added per gram wet weight of the cell pellet.
The bacterial pellet was thawed at 37°C in a water bath for 15 mins and kept on ice. The cells were lysed by sonication in a Branson Sonifier. Sonication was done at output 4 and 50% power with 3 cycles of 20 pulse bursts with a 10 sec pause between each cycle. The entire sonication was done on ice to prevent the heat generated during the process. The cell lysate were spun down at 14,000 rpm for 20 mins at 4°C. The supernatant was transferred to a fresh tube and the pellets (insoluble fraction) were stored for SDS-PAGE analysis. The clarified supernatant (soluble fraction) was stored separately for the SDS-PAGE analysis. The recombinant protein was first characterized by analyzing the soluble and insoluble fraction in SDS-PAGE. Both pET-32a+Trx+Moricin and pET-32a+Moricin recombinant proteins were found to be in the clarified fractions (soluble fraction). The supernatant was used for recombinant protein purification using native Immobilized Metal Affinity Chromatography.

4.3.2.6.2 PURIFICATION OF RECOMBINANT PROTEIN

The 6XHis tagged recombinant HpMoricin was purified by metal affinity chromatography using BD TALON™ Metal affinity resin from BD Biosciences. The recombinant protein was purified in native condition using the cobalt-based IMAC resin (Immobilized Metal Affinity Chromatography). The resin utilizes a special tetradeutate metal chelator for purifying the recombinant polyHistidine-tagged protein. The native protein purification procedure was adopted since the recombinant protein was identified in the soluble fraction. All the purification conditions were done at pH 7.0 to decrease the amount of nonspecifically bound proteins. The buffers used in native protein purification regimens preserves the three dimensional structure and surface charge characteristics of selected soluble protein during the harvest. Metal affinity chromatography column was prepared by applying 2 mL of BD TALON™ resin. The column was equilibrated twice with 10 mL of native lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF and 1 X Protease Inhibitor]
Cocktail for 10 mins. To the equilibrated column, 5 mL of supernatant after sonication was applied and mixed gently for 10 mins. The flow-through was allowed to pass through the column; another 5 mL of supernatant was applied to the column and mixed gently for 10 mins. The second flow-through was collected and stored for SDS-PAGE. The column was resuspended twice in 5 mL native wash buffer [50 mM Na₂HPO₄, 300 mM NaCl (pH 7.0)] containing 20 mM imidazole, mixed gently and the fractions were collected. Column was washed twice serially with 5 mL each of wash buffer containing 50 mM and 100 mM imidazole. Recombinant proteins were eluted from the column using native elution buffer [50 mM Na₂HPO₄, 300 mM NaCl (pH 7.0)] containing 150 mM imidazole. The protein fractions were analyzed spectrophotometrically and on a SDS-PAGE. Western blotting was carried out to confirm the elution profile of the recombinant protein.

4.3.2.7 Antimicrobial Assays

The activity of the recombinant moricin protein was checked by standard disc assay. The preliminary activity assay was done with the partial purified fractions. The E. coli strain was grown to an OD₆₀₀ in 0.8 mL in LB broth, then checked on LB agar plates and incubated for 37°C for an hour. Sterile discs (Himedia) were presoaked in 1 X PBS and dried. The partially purified fractions were applied in different concentration on presoaked sterile discs. A sterile forceps was used to place the discs on the LB agar plated with bacterial cells. The plates were incubated in 37°C for overnight. Antimicrobial activity of moricin was observed as a zone of clearance.

The recombinant HpDefensin peptide turned out to be lethal to the host expression strain BL21 (DE3) pLysS. Antibacterial assays were designed on the host expression system since the recombinant peptide inhibits the growth of host cells. The antibacterial activity was analysed indirectly by measuring the optical density of the induced culture at 600 nm using spectrophotometer. The pET-31b+ expression vector alone was
transformed into BL21 (DE3) pLysS cell and used as control. A single isolated colony was inoculated in the 2 mL LB broth with appropriate antibiotic and the culture was grown overnight at 37°C with 200 rpm in shaker cum incubator. From this overnight culture 500 µL was reinoculated in fresh medium for induction. The BL21 (DE3) pLysS cells were induced by 0.5 mM IPTG and culture were kept in a shaker cum incubator till an OD_{500-700} was obtained. The experiments were repeated thrice for reproducibility. The experiments done again to demonstrate the growth inhibition at OD_{1100-1200} to confirm endogenous action of induced recombinant peptide in BL21 (DE3) pLysS cells.
Table 4.1 - Primer sequences used for the amplification of *moricin* and *defensin* genes.

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Table 4.2 - Dataset used in the homology and phylogenetic analysis of *moricin* gene in order Lepidoptera.

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4.4 RESULTS

4.4.1 ISOLATION AND SEQUENCE CHARACTERIZATION OF AMP GENES

4.4.1.1 Moricin Genes

Post immunization of *H. puera* larva by *E. coli*, *HpMoricin I* and *HpMoricin II* genes were amplified from the cDNAs of *H. puera* larva using the gene specific primers. The cDNAs of *HpMoricin I* and *HpMoricin II* corresponded to 291 bp and 290 bp respectively (Figure 4.1). *HpMoricin I* and *HpMoricin II* isoforms were sequence characterized and the sequences were submitted to GenBank (AY847955 and AY847956). The partial cDNA sequences were translated and it was observed that *HpMoricin* peptide sequences contain partial signal peptide region which starting with 13th amino acid Ala^{13} in comparison with *BmMoricin*. The cDNA partial clones of *HpMoricin I* and *HpMoricin II* lack 12 amino acids in N-terminal of the signal peptide region. It was found that both *HpMoricin I* and *HpMoricin II* code for 54 amino acids corresponding to partial moricin gene.

The two moricin isoforms from the *H. puera* were synonymous isoforms has no amino acid change in the mature peptide region (Figure 4.2). The alignment of both moricin I and moricin II isoforms of *H. puera* are shown in the figure 4.2. *HpMoricin I* and *HpMoricin II* differ by three transversions that were found after the stop codon positions (168C→G, 274A→C and 279G→T). *HpMoricin I* and *HpMoricin II* have 97% sequence similarity at the nucleotide level while 100% similarity was found between the *HpMoricin* and *BmMoricin*. Since the coding region had 100% similarity with the already existing peptides, we proceeded further in screening other novel peptides from *H. puera*.

4.4.1.2 Defensin Gene

The defensin genes were amplified from the cDNAs of *H. puera* larva using the defensin specific primers designed based on the *S. calcitrans* sequences. Defensin gene was amplified, cloned in pGEM^{®}-T Easy vector and sequence characterized. A 263 bp encoding the full length
defensin gene was amplified from the cDNA of immunized larva of H. puera (Figure 4.3). The cDNA clone was sequenced and submitted to NCBI GenBank (EF534713). This forms the first report of defensin gene from Lepidoptera which resembles the dipteran defensin. HpDefensin gene contains an ORF of 263 bp which codes for 79 amino acids showing the typical signature of six cysteines in the mature peptide region (Figure 4.4).

4.4.2 In silico Characterization of Moricin and Defensin

Based on the translated protein sequences from the cDNA sequences of HpMoricin and HpDefensin peptides, the phylogenetic trees were constructed using NJ algorithm with Poisson correction model. The correlation of the nearest neighbourhood of H. puera based on the mature peptide sequences along with structural homology one can predict the functionality or the functional diversity in insect phylogeny.

4.4.2.1 Phylogenetic Analyses of Moricin and Defensin

Moricin is a unique antimicrobial peptide which is reported exclusively from order Lepidoptera. The dataset for the phylogenetic studies contained both cDNA and protein sequences from the GenBank (Table 4.2). In the current study the sequence of the matured peptide from H. puera was used since the cDNA clones were partial. The multiple alignments were done using BioEdit v 7.0.5.3 and the conserved amino acids were highlighted. The potential sites involved in the structural motifs formation are highlighted on the multiple alignments (Figure 4.5). The NJ tree of HpMoricin peptide shows that it forms the cluster with B. mori and A. pernyi leaving the rest of moricin sequences in the separate cluster (Figure 4.5). When correlating the structural data with the phylogenetic data it was observed that moricin sequences from B. mori, H. puera and A. pernyi had two \( \gamma \)-turns which was flanking the
α-helix. The first γ-turn was contributed by $A^1 K^2 I^3$ ($A^1$, Lys$^2$ and Ile$^3$) and second γ-turn was contributed by $K^{39} R^{40} K^{41}$ (Lys$^{39}$, Arg$^{40}$ and Lys$^{41}$) amino acids. While, the second cluster housing the $H$. virescens, $M$. sexta, $S$. littura and $S$. exigua moricin shows two consecutive β-turns located at the C-terminal end of α-helix.

Defensin peptides have been isolated from wide variety of species from invertebrates to vertebrates. The dataset for the defensin peptides contain mostly the translated cDNA sequences and few protein sequences pertaining to class Insecta. Some of the synonymous isoforms were also included for the study. The multiple alignments were carried out and the aligned sequences were manually checked for gaps and misalignment (Figure 4.6 and 4.7). The phylogenetic analysis was done for the dataset of 27 tax and NJ trees were constructed using Poisson correction model for the signal peptide and mature peptide regions separately. The phylogenetic analysis based on the signal and mature peptides resulted in more or less similar observation, where all the major clusters remained intact (Figure 4.6 and 4.7). However, in case of dipterans the phylogeny of the mature peptides bifurcated into two distinct clusters, while in the signal peptide they clustered together. $H$. puera clustered with the Stomoxys calcitrans (stable fly) and between these two species the percentage similarity of the peptide sequences was around 98%.

4.4.3 STRUCTURAL CHARACTERIZATION OF MORICIN AND DEFENSIN

4.4.3.1 ISOELECTRIC POINT OF AMPs

The theoretical mass and the isoelectric points (pI) were predicted for the mature peptide based on the amino acid sequences of moricin and defensin peptides (Table 4.2 and 4.3). The theoretical isoelectric point of moricin did not vary much between neighbours (10 to 11). In the case of defensins the isoelectric point for HpDefensin was observed to be the least 5.98 which was followed by $S$. calcitrans defensins smd1
RESULTS

(6.61) and smd2 (7.55). The pl values for rest other members in the defensin dataset fell in between 8 to 10 (Figure 4.8).

4.4.3.2 HOMOLOGY MODELING OF AMPs

The raw sequences of the moricins and the HpDefensin were fed into Swiss-Pdb viewer and the appropriate template for the homology modeling was searched from the Expdb templates database. The resultant hits were screened for its resolution and sequence similarity with the translated protein sequences for both the AMPs. HpMoricin gene showed 100% similarity with BmMoricin sequence (PDB ID-1KV4) and 72.5% similarity with the SMoricin sequence (PDB ID-1X22). Since 100% similarity was found for HpMoricin with BmMoricin, the homology modeling for H. puera moricin was less meaningful. Therefore, the homology models were generated for rest other moricins based on their clustering pattern. BmMoricin (PDB ID-1KV4) was used as a template for generating ApMoricin model (DQ519400) which has 99% similarity. While SMoricin (PDB ID-1X22) was employed as a template for modeling HvMoricin (P83416), MsMoricin (AY232301) and SeMoricin (AY611631) sequences.

In spite of many accession records found in the GenBank database for defensin peptide, only few of insect defensin crystal structures were available in PDB server. This becomes a limiting factor in template selection. Insect defensin A from Phormia terranovae (PDB ID-1ICA) was selected as it had 60.5% sequence similarity with HpDefensin.

4.4.3.3 VALIDATION OF MODELS

Robust models of moricin and defensin peptides were computed by using Deep View swiss-Pdb viewer v 3.7 homology modeling software. PROCHECK online software was employed to verify for stereochemical quality of the completed model. The generated models were then validated online in PDBsum (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum). Moricin homology model was generated using 1KV4 and 1X22 templates.
for A. pernyi, H. virescens and M. sexta. Ramachandran plots were generated to measure the strength of the conformational angles of the residues for both template and for the generated models using Molsoft ICM and PROCHECK online software (Figure 4.9, 4.10 and 4.11). The results of Ramachandran plots show that all the moricin peptides have 80% of the residues in favourable region. Ramachandran plot analysis for the defensin template (1ICA) was found to have 67.7% residues were in the most favored regions (Figure 4.11). While for HpDefensin the analysis showed 63.6% residues in the most favored regions, 33.3% in the additional allowed regions and none in the generously allowed regions and one residue L (Leu6) in the disallowed regions. The plot statistics of the Ramachandran plots are shown in a table along with respective plots.

4.4.3.4 Predicted Structure

The amino acid sequences of HpMoricin reveal the basic nature of the peptides. The basic nature of the peptide was contributed by Lysine (K) and Arginine (R). These amino acids are highlighted on the back bone of the linear peptide figure 4.12A*. The secondary structure shows an α-helix extending from Pro4-Leu35 and two γ turns flanking the helix was contributed by Ala1, Lys2, Ile3 and Lys39 Arg40 Lys41 respectively. The electrostatic potential surface view, charge and polar surface view of H. puer a moricin is shown in figure 4.12A, 4.12B and 4.12C. The 180° rotation of the surface view is shown in figure 4.12B* and 4.12C* for the better understanding. The detailed graphical representation of the secondary motifs along with their corresponding amino acid sequences were highlighted for the moricins in figure 4.13. Based on the structure and phylogeny, the moricin peptides can be further classified into two types based on their turns. The first type of moricin has two γ-turns flanking the α-helix and the second type of moricin have two β-turns found in the C-terminal of α-helix. The maximum parsimony (MP) tree based on the
mature peptide sequences clearly distinguishes the clustering of moricin based on structural similarities (Figure 4.14).

In defensin, the predicted secondary structure shows an α-helix followed by two typical antiparallel β-strands with β-turn. The significant three disulfide bridges formed by the six cysteines are highlighted on the homology modeled HpDefensin (Figure 4.15A). In HpDefensin the bridges were formed between cysteine molecules at C\textsuperscript{42}-C\textsuperscript{69}, C\textsuperscript{55}-C\textsuperscript{75} and C\textsuperscript{59}-C\textsuperscript{77} positions. The helix is contributed by ten amino acids (Ala\textsuperscript{53}-Lys\textsuperscript{62}) followed by two antiparallel β-strands contributed by (Tyr\textsuperscript{68} and Cys\textsuperscript{69}), (Cys\textsuperscript{75} and Val\textsuperscript{76}) which is interconnected by β-turn contributed by (Thr\textsuperscript{70}, Lys\textsuperscript{71}, Glu\textsuperscript{72} and Gly\textsuperscript{73}) amino acids. The electrostatic potential surface view, charge and polar surface view of H. puera defensin are also shown in figure 4.15B and 4.15C. The negatively charged amino acids Asp\textsuperscript{43}, Asp\textsuperscript{64}, Glu\textsuperscript{72} and Glu\textsuperscript{79} are demarcated in red. The detailed graphical structures explaining the secondary structural motifs are shown in figure 4.16.

### 4.4.4 FUNCTIONAL CHARACTERIZATION OF Moricin AND Defensin

#### 4.4.4.1 Transcript Analysis

The semi quantitative RT-PCR was carried out to quantitate the mRNA transcript of moricin gene. For this, the expression level of moricin I mRNA transcript was estimated indirectly by PCR. The cDNA prepared from 0 hr, 2 hrs, 4 hrs, 6 hrs, 12 hrs, 24 hrs and 48 hrs post immunized larval samples were used as template for quantifying the moricin gene transcript. For this purpose 28SrRNA was used as housekeeping gene to normalize the moricin gene expression (Figure 4.17). The PCR products were visualized in the 1.5% agarose gel and the densitometric analysis of RT-PCR products were carried out by normalizing the products with internal control using Quantity one software module in Fluor-S\textsuperscript{TM} multi imager system (Bio-Rad).

The semi quantitative RT-PCR data suggest the inducibility of HpMoricin gene as an immune response due to E. coli infection. This
result is also supported by the control template where the mRNA of *moricin* transcript was not amplified confirming the microbial inducibility of the *moricin* gene in *H. puera* larva. The expression level of the *moricin* transcript was found to reach its maximum at 24 hrs and then start declining as observed at 48\textsuperscript{th} hr of post infection. The graphs were plotted based on the densitometric values obtained from Quantity one software (Figure 4.18). The results suggested a time dependent increase in the *moricin* transcripts level up to 24 hrs followed by the decrease in the expression level.

### 4.4.4.2 Translational Analysis

#### 4.4.4.2.1 Expression of *Moricin* Peptide

*HpMoricin* was sub-cloned from the pGEM\textsuperscript{®}-T vector to pBluescript SK vector due to unavailability of compatible restriction sites in the pET-32 expression vector. *Spe* I and *Sac* II sites were selected and restriction digestion was carried out to release the *moricin* cDNA insert from the pGEM\textsuperscript{®}-T vector and then cloned in pBluescript SK (Figure 4.19). From the recombinant pBluescript SK vector the insert was released by *BamH* I and *Sac* I enzymes and cloned into the pET-32a expression vector (Figure 4.20). Colony hybridization was carried out to pick the positive clone (Figure 4.21). Further the colonies were screened for the orientation by colony PCR using vector-specific primer (S.tag) with gene-specific reverse primer (Morrev1). In the case of negative clones (i.e.) the self ligated or the reverse oriented clones the PCR products were not formed (Figure 4.22). Final confirmatory method was done by sequencing the positive clone to ensure that the *HpMoricin* in frame with the expression vector. The schematic diagram of the vector map with the highlighted restriction sites utilized for the experiment is shown in Figure 4.23A. The *thioredoxin* tag was released from the pET-32a+Trx+*Moricin* construct using *Nde* I digestion (Figure 4.23B).
The linearized construct was self-ligated after the removing the thioredoxin tag (Figure 4.23C).

The expression of *HpMoricin* was carried out in JM109 (DE3) expression cells under standardized condition as mentioned above. The recombinant fusion protein was separated in 12% SDS-PAGE along with prestained protein marker (Figure 4.24). The size of the fusion protein was around 27 kDa of which 20 kDa was contributed by the vector tags. The western blotting was carried out using mouse monoclonal anti-polyhistidine antibody to confirm that the expressed fusion protein is histidine tagged (Figure 4.25). *Moricin* without thioredoxin tag was also expressed and was separated in 15% SDS-PAGE (Figure 4.26). The immunoblotting experiment using monoclonal anti-polyhistidine antibody confirms the presence of the recombinant moricin peptide (Figure 4.27). But the expression level was comparatively low, when compared to Trx+Moricin fusion protein.

The recombinant fusion protein was induced in large scale and the expression was optimized as mentioned in the methods. Different fractions were collected during the purification process and stored for the SDS-PAGE analyses. The recombinant moricin was observed to get eluted at 50 mM concentration of Imidazole. The purification experiments were standardized for the recombinant moricin by decreasing the Imidazole concentration in the wash buffer to 20 mM and followed by 40 mM wash. Elution buffer contained 50 mM and 100 mM concentration of Imidazole (Figure 4.28). Western blot was carried out for the eluted fractions to confirm that purified proteins are histidine tagged (Figure 4.29). A preliminary growth inhibitory zone assay was carried out with sterile disc (Himedia) containing different concentration of eluted fractions of recombinant moricin to show the preliminary antibacterial activity in the JM109 *E. coli* cells (Figure 4.30).
4.4.4.2 Expression of Defensin Peptide

 HpDefensin peptide was amplified from the cDNA clone with modified forward primer containing Nde I restriction site followed by 18 nucleotides coding for 6XHis amino acid at the 5' region and the 3' region had gene specific sequence. The reverse primer contained the Xho I site. The PCR products were incorporated with Nde I and Xho I site so that it can facilitate directly cloning into pET-31b+ expression vector. Additionally, another primer pair was synthesized to get the C-terminal His-tagging in which the forward primer contained only the Nde I site and reverse primer contain Xho I site with deleted stop codon so that the vector His tag can be utilized (Table 3.1). Both N-terminal tagging and C-terminal tagging were thus obtained by PCR using primers (Figure 4.31).

These PCR products were cloned again into the pGEM®-T easy vector and the restriction digestion were carried out using Nde I and Xho I enzymes to ensure the restriction site were intact after PCR amplification (Figure 4.32). The digested products were purified and cloned into the pET-31b+ expression vector. The plasmids were isolated from positive clones and the clones were checked again by double digestion for insert release (Figure 4.33C). The positive clones were sequenced to check their respective frame with the vector ATG. The schematic sketch of the vector map and the procedure are depicted in the figure 4.33 and 4.34.

Induction of HpDefensin was standardized at 28°C with 0.5 mM IPTG concentration. Interesting results were observed, when HpDefensin clones were expressed in the pET-31b+ expression system. When the recombinant peptide was induced by IPTG, the growth of the host expression strain BL21 (DE3) pLysS was inhibited. Assay for antibacterial activity was designed to analyze the growth inhibition in the host cells itself. Both N-terminal 6XHis and C-terminal 6XHis defensins shows activity by inhibiting the growth of BL21 (DE3) pLysS expression
strain (Figure 4.35). The graphs were plotted with OD at different intervals of time along with the positive control i.e. pET-31b+ vector transformed in BL21 (DE3) pLysS (Figure 4.36). The experiment was repeated with slight modification by inducing the expression strain at higher OD_{1100-1200} and the observations confirm that the growth of the BL21 (DE3) pLysS cells were arrested after induction by IPTG (Figure 4.37). The growth of the BL21 (DE3) pLysS cells gets arrested or inhibited and the OD remains constant which further confirms that the defensin peptide has endogenous action which interfere with cell division of *E. coli* bacterium.
Figure 4.1 - Agarose gel picture showing the RT-PCR product of *HpMoricin* isoforms.

M - 100bp ladder (NEB), Lane 1 - *HpMoricin I* and Lane 2 - *HpMoricin II*.

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Figure 4.2 - Sequence alignment of *HpMoricin I* (291 bp) and *HpMoricin II* (290 bp) isoforms along with coding amino acids.

Arrows indicate the differences in the nucleotide sequences.
Figure 4.3 - Agarose gel picture showing the amplification of *HpDefensin* gene from the cDNA of the immunized larva.
Lane M - 1kb plus ladder (*Invitrogen*), lane 1 and 2 shows the amplified RT-PCR product.

Figure 4.4 - Translated Sequence of *HpDefensin* showing the longest Open Reading Frame (ORF).
Arrows indicate the start, stop and six cysteines.
Figure 4.5 - NJ tree on right and the corresponding multiple alignment of *moricin* peptides showing conserved region. The predicted potential sites for the secondary motifs are highlighted on the sequences.
Figure 4.6 - NJ tree with corresponding multiple alignment of the signal peptide region of *defensin* gene in insects. The highlighted regions in green are identical amino acids and yellow are similar amino acids in majority sequences.
Figure 4.7 - NJ tree with corresponding multiple alignment of the mature peptide regions highlighting the conserved region. The secondary predicted motifs are highlighted on the sequences.
Figure 4.8 - Comparative graph showing the isoelectric point (pI) of defensins in different taxa.
Figure 4.9 - Ramachandran plot showing the amino acid residues plotted in Phi and Psi degrees for template (left), *ApMoricin* (right) and plot statistics is tabulated.

The highlighted leucine residue falls in the disallowed region.

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Figure 4.10 - Ramachandran plot showing the amino acid residues plotted in Phi and Psi degrees for template SI/Moricin (1X22), HvMoricin, MsMoricin and plot statistics is tabulated.

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Figure 4.11 - Ramachandran plot showing the amino acid residues plotted in Phi and Psi degrees for template (left), HpDefensin (right) and plot statistics is tabulated. The highlighted leucine residue falls in the disallowed region.

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<td>5</td>
<td>5</td>
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<tr>
<td>Number of proline residues</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>100%</td>
<td>40</td>
<td>100%</td>
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Figure 4.12 - Predicted three dimensional structure of *HpMoricin* using Swiss PDB viewer.

Figure A showing the secondary structure, A* highlights the basic amino acids, B shows the surface electrostatic potential view of *moricin* orientated as in A, colored according to electrostatic potential with positive regions in blue and negative in red and C shows the amino acid charges and polarity. B* and C* shows the rotated view by 180° about the vertical axis.
Figure 4.13 - Graphical representation of the secondary structures of homology modeled mature peptide region of moricin genes showing the motifs. The 1KV4 and 1X22 are the templates used for modeling moricin genes.
Figure 4.14 - Circular MP tree showing different moricin structures with highlighted amino acid conversions from their corresponding templates 1KV4 and 1X22.
Figure 4.15 - Predicted three dimensional structure of HpDefensin using homology modeling.

Figure A showing the secondary structure, B shows the surface electrostatic potential view of defensin orientated as in A, colored according to electrostatic potential with positive regions in blue and negative in red and C shows the amino acid charges and polarity. B* and C* shows the rotated view by 180° about the vertical axis.
Figure 4.16 - Graphical representation of the structural motifs of mature peptide region of defensin genes.
The ica was the template used to model H. puera defensin.
Figure 4.17 - Semiquantitative RT-PCR showing the expression of *HpMoricin I* gene transcripts after immunization with *E. coli*.

Lane 1 - negative control, lane 2 - control, lane 3 - 8 shows the expression pattern of *HpMoricin* transcript in different time intervals after post-immunization lane 3 - (2 hrs), lane 4 - (4 hrs), lane 5 - (6 hrs), lane 6 - (12 hrs), lane 7 - (24 hrs) and lane 8 - (48 hrs).

<table>
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<th>28SrRNA (179.901)</th>
<th>28SrRNA (208.650)</th>
<th>28SrRNA (208.420)</th>
<th>28SrRNA (200.960)</th>
<th>28SrRNA (204.660)</th>
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</thead>
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<td>18.740</td>
<td>19.430</td>
<td>30.860</td>
<td>64.620</td>
<td>91.850</td>
<td>57.810</td>
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</table>

Figure 4.18 - Graphical representation of densitometric analysis of *HpMoricin I* RT-PCR products.

The plotted values are mean intensity obtained after blank normalization using Quantity One software.
Figure 4.19 - Schematic representation of the vector map of pGEM®T highlighting the restriction sites used for releasing the moricin fragment (A). Agarose gel picture showing corresponding enzymatic release of moricin (B).

Lane M - 100bp ladder (NEB) and lane 1 - vector backbone and insert.
Figure 4.20 - Schematic representation of pBluescript II SK+ vector map showing the restriction sites (A), Agarose gel picture showing the insert release using BamH I and Sac I enzymes (B).
Lane M - 100bp ladder (NEB) and lane 1 shows released insert and vector backbone.
Figure 4.21 - Autoradiogram of colony hybridization showing the positive colonies. 
*HpMoricin* gene in the pET-32a expression vector.

Figure 4.22 - Agarose gel showing the colony PCR products, amplified using *S.tag* primer with *moricin* reverse primer.
Lane M - 1 kb plus ladder (Invitrogen), Lane 1 - negative control, lane 2 - negative colony and lane 3 - positive colony showing the amplification.
Figure 4.23 - Schematic representation of the vector maps of pET-32a+moricin construct.

(A) Map of pET-32a+Trx+Moricin construct, (B) Agarose gel picture showing the linearized pET-32a+Moricin construct using NdeI enzyme and (C) the vector map of pET-32a+Moricin construct where thioredoxin tag is removed.
Figure 4.24 - SDS-PAGE showing the expressed recombinant *moricin* fusion protein in JM109 (DE3) *E.coli* cells.
Lane M - Marker (NEB), lane 1 - uninduced pET-32a vector alone (0 hr), lane 2 - induced pET-32a vector (2 hrs), lane 3 - pET-32a vector (4 hrs), lane 4 - uninduced pET-32a+Trx+Moricin (0 hr), lane 5 - induced pET-32a+Trx+Moricin (1 hr), lane 6 - induced pET-32a+Trx+Moricin (2 hrs), lane 7 - induced pET-32a+Trx+Moricin (3 hrs) and lane 8 - induced pET-32a+Trx+Moricin (4 hrs).

Figure 4.25 - Western blot showing the expressed recombinant *moricin* fusion protein using monoclonal anti-polyhistidine antibody.
Lane M - Prestained Marker (NEB), lane 1 - uninduced pET-32a vector alone (0 hr), lane 2 - induced pET-32a vector alone (2 hrs), lane 3 - induced pET-32a vector alone (4 hrs), lane 4 - uninduced pET-32a+Trx+Moricin (0 hr), lane 5 - induced pET-32a+Trx+Moricin (1 hr), lane 6 - induced pET-32a+Trx+Moricin (2 hrs), lane 7 - induced pET-32a+Trx+Moricin (3 hrs), lane 8 - induced pET-32a+Trx+Moricin (4 hrs) and lane 9 - induced pET-32a+Trx+Moricin (5 hrs).
Figure 4.26 - SDS-PAGE showing the expressed recombinant moricin fusion peptide in JM109 (DE3) E.coli cells.
Lane M - Marker (NEB), lane 1 - uninduced pET-32a vector alone (0 hr), lane 2 - induced pET-32a vector (2 hrs), lane 3 - pET-32a vector (4 hrs), lane 4 - uninduced pET-32a+Moricin (0 hr), lane 5 - induced pET-32a+Moricin (1 hr), lane 6 - induced pET-32a+Moricin (2 hrs), lane 7 - induced pET-32a+Moricin (3 hrs), lane 8 - induced pET-32a+Moricin (4 hrs) and lane 9 - induced pET-32a+Moricin (5 hrs).

Figure 4.27 - Western blot showing the expressed recombinant moricin fusion protein using monoclonal anti-polyhistidine antibody.
Lane M - Prestained Marker (NEB), lane 1 - uninduced pET-32a+Moricin (0 hr), lane 2 - induced pET-32a+Moricin (1 hr), lane 3 - induced pET-32a+Moricin (2 hrs), lane 4 - induced pET-32a+Moricin (3 hrs), lane 5 - induced pET-32a+Moricin (4 hrs) and lane 6 - induced pET-32a+Moricin (5 hrs).
Figure 4.28 - Purification profile of recombinant moricin using His-affinity column chromatography.
Lane M - Marker, lane 1 - pET-32a vector alone induced, lane 2 - Crude lysate of pET-32a+Trx+Moricin, lane 3 - sonicated lysate, lane 4 and 5 - Fractions of 20 mM Imidazole wash, lane 6-9 Fractions of 50 mM Imidazole, lane 10-13 fractions of 100 mM Imidazole, lane 14 - 150 mM Imidazole

Figure 4.29 - Western blot for the purified fractions using monoclonal anti-polyhistidine antibody to confirm the eluted recombinant protein.
Lane M - Marker, lane 1 - Crude lysate, lane 2 - 5 were fractions of 50 mM Imidazole, lane 6 - 8 were fractions of 100 mM Imidazole
Figure 4.30 - Antibacterial disc assay showing the zone formation in *E. coli* by the partial purified fractions of recombinant *thioredoxin-moricin* peptide.

CO- Control crude lysate of induced thioredoxin tag, a- 5 μL, b- 10 μL, c- 15 μL and d- 20 μL of partial purified *moricin*. 
Figure 4.31 - Agarose gel showing the recombinant defensin PCR products. Lane M - 100 bp ladder (NEB), lane 1 - N-terminal-6XHis defensin and lane 2 - C-terminal-6XHis defensin.

Figure 4.32 - Agarose gel picture showing the expected insert release using NdeI and XhoI enzymes. Lane M - 1kb plus ladder (Invitrogen), lane 1 - N-terminal 6XHis-defensin, lane 2 - C-terminal defensin and lane 3 - negative colony
Figure 4.33 - Schematic representation of the vector maps showing the double digestion (A & B), Agarose gel picture showing the double digestion profile of positive clone using *Nde I* and *Xho I* (C).

Lane M - 100bp ladder, lane 1 and 2 N-terminal 6XHis-defensin, lane 3 - C-terminal defensin and lane 4 - pET-31b+ vector showing the KSI release.
Figure 4.34 - Schematic representation of the defensin constructs in pET-31b+ expression system showing the N and C-terminal 6XHis tagging.
Figure 4.35 - Demonstration of antibacterial action of recombinant *defensin* peptide in host expression strain BL21 (DE3) pLysS.

Panel A shows pET-31b+N-6XHis+*Defensin* and panel B shows pET-31b+*Defensin+C*-6XHis recombinant peptides showing the optical density (OD) clearance in BL21 (DE3) pLysS host expression *E.coli* strain after induction with IPTG. 1-(0hr), 2-(2hrs), 3- (4hrs), 4-(6hrs) and 5-pET-31b+ vector after (6hrs) of post induction.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
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<th>DEF-C</th>
<th>pET31b+</th>
</tr>
</thead>
<tbody>
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<td>0.42</td>
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<tr>
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<tr>
<td>10</td>
<td>0.72</td>
<td>0.65</td>
<td>1.79</td>
</tr>
</tbody>
</table>

Figure 4.36 - Antibacterial activity graph plotted based on the optical density showing the lethality by *HpDefensin* gene in host expression strain BL21 (DE3) pLysS.
Figure 4.37 - Antibacterial activity graph plotted based on the optical density showing the lethality caused by *HpDefensin* gene in BL21 (DE3) pLysS. Induction was done at OD$_{1100-1200}$. 
4.6 DISCUSSION

Moricin was first isolated from the hemolymph of *B. mori* and had no significant resemblance with any of the existing families of antimicrobial peptides (Hara and Yamakawa, 1995). Moricin is a linear peptide, highly basic in nature. The basicity is contributed by the predominant distribution of basic amino acids such as Lysine (Lys\(^2\), Lys\(^6\), Lys\(^9\), Lys\(^{13}\), Lys\(^{17}\), Lys\(^{36}\), Lys\(^{38}\), Lys\(^{39}\), Lys\(^{41}\)) and Arginine (Arg\(^{20}\), Arg\(^{40}\)). The higher basicity tends to correlate with higher antibacterial activities (Hoffmann and Hetru, 1992; Cociancich *et al.*, 1994a). The C-terminal region of moricin is more hydrophobic, the surface potential of the \(\alpha\)-helix from Asn\(^{23}\) to Lys\(^{36}\) are almost hydrophobic except Asp\(^{30}\) which is negatively charged (Hemmi *et al.*, 2002). The electrostatic map shows largely positively charged and was contributed by the cluster of four basic residues (K\(^{36}\), K\(^{38}\), K\(^{39}\), R\(^{40}\) and K\(^{41}\)). The basicity also responds to the electrostatic interaction between the positively charged peptide and the negatively charged bacterial surface (Christensen *et al.*, 1988; Gabay, 1994).

The N-terminal half of the moricin contains charged amino acids that appear at equal intervals of three or four amino acid residues, indicating the amphipathic \(\alpha\)-helix that structurally corresponds to the antibacterial peptide (Cociancich *et al.*, 1994a; Kreil, 1994). The distribution of the amino acid clearly demarcates hydrophobic and hydrophilic faces which also counter supports the presence of an amphipathic \(\alpha\)-helix. The isoelectric point of matured moricin peptide was calculated and its pl ranges from 10 to 11 which were comparatively higher than any other AMP families (Table 4.2). In insect *defensin* the \(\alpha\)-helical region is reported to be responsible for both antibacterial activity and increased membrane permeability (Yamada and Natori, 1994). The antibacterial activity of the moricin peptide has been successfully demonstrated against *E. coli*, *Acinetobacter* sp., *Pseudomonas fluorescens*, *P. aeruginosa*, *Bacillus subtilis*, *B. megaterium*, *B. cereus*, *Staphylococcus aureus*, *S. xylosus*, *S. epidermidis* and *S. pyogenes* (Hara and Yamakawa, 1995).
**Moricin** unlike most other similar AMPs has no modified amino acid residues such as the α-amidation, hydroxylation, O-glycosylation or disulfide bridge formation. **Cecropins** which are linear peptides like moricin with two α-helices linked by a short hinge are frequently C-terminally amidated and hydroxylation of Lysine residues have been reported in *B. mori* (Steiner et al., 1981; Cociancich et al., 1994b; Bulet et al., 1993). Similarly, **Lebocins** which are proline-rich peptides and has the unique threonine residue is often O-glycosylated (Hara and Yamakawa, 1995; Furukawa et al., 1997). Moricins are devoid of cysteines which are responsible for forming intramolecular disulfide bonds in defensins (Kuzuhara et al., 1990; Lepage et al., 1991). This unique property of moricin makes them ideal for synthesis either chemically or biologically using suitable prokaryotic expression system.

The phylogenetic analysis of moricins isolated from different lepidopterans clearly shows two distinct clusters in spite of significant conserved amino acids. While correlating the structural data along with the phylogenetic data a clear demarcation of two clusters were observed by the presence of γ and β-turns. The first cluster contains *B. mori*, *H. puera* and *A. pernyi* has two γ-turns which inter-flanks the α-helix while the second cluster hosting *H. virescens*, *S. litura*, *S. exigua* and *M. sexta* has two β-turns found at the C-terminal of the α-helix.

**Defensins** are small 3-4 kDa, cationic molecules, with six cysteines engaged in three intra-molecular bridges (Lambert et al., 1989). Their presence has been reported from many insect orders and also from scorpions and molluscs (Ehret-Sabatier et al., 1996; Charlet et al., 1996). It was first reported from cell culture flasks in *Sacrophaga peregrine* (Matsuyama and Natori, 1988) but later it was isolated experimentally from injured larva of *P. terranovae* (Lambert et al., 1989). From order Lepidoptera no defensins has been reported so far, however, defensin-like peptides were isolated from lepidopterans such as *Heliomicin* from *H. virescens* and had
demonstrated for antifungal activity against the filamentous fungi (*Aspergillus fumigatus*, *Fusarium culmorum*, *F. oxysporum*, *Nectria hematococca*, *Neospora crassa* and *Trichoderma viride*) and yeast strains (*Candida albicans*, *Cryptococcus neoformans*), while it lacked antibacterial activity (Lamberty et al., 1999). In contrast, *HpDefensin* show antibacterial activity against *E. coli* cells. Besides *HpDefensin* lacked sequence similarity with other defensin-like peptides from lepidopterans. The phylogenetic analysis of defensins with the signal peptide region and the mature peptide region shows *HpDefensin* is similar to dipteran defensins. Since the mature peptide is structurally significant, the alignment of signal and mature peptide region were scored separately. The comparison of structural models of defensins could not be generated due to lack of sequence homology between different defensins falling in different clusters.

Defensins are a family of cationic peptides that includes the phormicins, sapecins, royalisin and spodoptericin (Volkoff et al., 2003). They are 38 to 45 amino acids long, and consist of an α-helix linked by a loop to an antiparallel β-sheet (Hanzawa et al., 1990; Bonmatin et al., 1992). These peptides undergo post translational modification, and the six cysteines which form disulphide bridges between the 1-4, 2-5, 3-6. The N-terminal extended defensin has been reported in the gut of the stable fly (Lehane et al., 1997) while C-terminal extended defensins have been reported in the bumblebee (Hymenoptera) and in the royal jelly of the honeybee (Rees et al., 1997; Saltykova et al., 2005). In contrast to cecropins that are frequently C-terminal amidated, only two defensins (royalisin and bumblebee defensin) were found to be amidated at their C-terminus (Fujiwara et al., 1990).

Defensins are primarily active against G+ve and G-ve bacteria, but various mammalian defensins have been reported to show significant *in vitro* activity against fungi (Lehrer and Ladra, 1977; Ganz et al., 1985) and enveloped viruses (Lehrer and Ladra, 1977; Daher et al., 1986).
respect to their *in vitro* activity against bacteria or filamentous fungi, *defensins* can be classified in two sub-families: antibacterial *defensins* that stop preferentially the growth of bacteria and antifungal *defensins* that are predominantly effective against filamentous fungi (Bulet and Stöcklin, 2005). Indeed only few antifungal *defensins* have been reported (Bulet et al., 2003). *Drosomycin* from *Drosophila* (Michaut et al., 1996), two *defensin* like peptides were reported from the lepidopterons, *Heliomicin* from *H. virescens* and *galiomicin* from *Galleria mellonella* (Lamberty et al., 1999; 2001a; Schuhmann et al., 2003), *termicin* from the isopteran *P. spiniger* and Alo13 from the coleopteran *Acrocinus longimanus* (Barbault et al., 2003). Most of the insect *defensins* are demonstrate antibacterial activity. Antibacterial *defensin* are reported to be isolated from many dipterans, like *P. terranovae, Aedes aegypti, Anopheles gambiae, Chironomus plumosus, G. mellonella* (Dimarcq et al., 1990; Cho et al., 1996; Richman et al., 1996; Lauth et al., 1998; Cytrynska et al., 2007) from coleopteran like *Zophobas atratus, Allomyrina dichotoma, Anomala cuprea* (Bulet et al., 1991, Miyanoshita et al., 1996; Yamauchi, 2001). In case of hymenopterans *defensins* were isolated from *Bombus pascuorum, Apis mellifera, Formica rufa* (Rees et al., 1997, Fujiwara et al., 1990; Taguch et al., 1998), in Hemipteran from *Pyrrhocoris apterus, Rhodnius prolixus* (Cociancich et al., 1994; Lopez et al., 2003), in Thysanuran from *Thermobia domestica* (Altincicek and Vilcinskas, 2007).

The NMR studies on the three dimensional solution structures of *defensin* peptides in this family were obtained in aqueous solution (Bonmatin et al., 1992; Cornet et al., 1995; Hanzawa et al., 1990). *Phormia* and *Sarcophaga defensins* (Maget-Dana et al., 1995; Takeuchi et al., 2004) and of four antifungal *defensins* (*drosomycin, heliomicin, termicin* and Alo3) have been solved on recombinant molecules by 1H-NMR spectroscopy (Bulet et al., 2003; Thevissen et al., 2004). The structure of the antibacterial *defensins* and termicin can be described as an α-helical
domain and two antiparallel β- strands stabilized by two disulfide bridges (αββ) (Da Silva et al., 2003). For helomicin and drosomycin an additional scaffold is presented by another short N-terminal β-strand (ββββ) (Lamberty et al., 2001b; Landon et al., 1997). The typical and unique triple-stranded β-sheet scaffold of insect defensins (βββ) has been observed for Alo3 isolated from the harlequin beetle. A second interesting finding is that Alo3 has all the structural characteristics of bacterial growth inhibitors of the cystine-knot family (Thevissen et al., 2004).

Insects are the most primitive species which started evolving from Devonian period and their immune system had been exposed to tremendous amount of selection pressure exerted by the environment. This in turn made them an ideal model organism for discovering various novel AMPs. In the present study, HpMoricin and HpDefensin were isolated and characterized. However, the current study is limited to preliminary observation on AMP activity, but Insilco approaches can be used to identify and characterize various other AMPs from different insects, before initiating a large scale production. In future the study will help us in understanding the underlying mechanism that associate with AMP activity in wide spectrum of organisms.
4.6 SUMMARY

In the present study, two important immune related defense genes were identified for the first time from *H. puera*. The study involved identification, isolation and characterization of *Moricin* and *Defensin* genes from the immunized *H. puera* larvae. The phylogenetic analyses revealed presence of two major clusters of *moricin* which was further explained by phylo-structural analyses. The *moricin* peptide showed two structural motifs, one with γ-α-helix-γ turns and other with α-helix-β-β turn motifs. The sequence and structure of mature peptide region *HpMoricin* mimics the γ-α-helix-γ *moricin* type of *B. mori*. While comparison with similar species was not possible for *defensin* gene though *defensin*-like peptides are reported in lepidopteran which has less similarity with *HpDefensin*. However, sequence and the phylogenetic analyses revealed that *HpDefensin* belongs to αββ *defensin* type and it clusters with *S. calcitrans*. The structural analyses showed that the mature peptides form an α-helix followed by two antiparallel β-strand with four β-turns. The secondary structure of *HpDefensin* shows three disulphide bridges which was formed by six cysteines and the bonding order was C$^1$-C$^4$, C$^2$-C$^5$ and C$^3$-C$^6$. These preliminary experiments with *moricin* and *defensin* AMPs show antibacterial activity with *E. coli* cells. This study provides a novel idea of correlating the structural data with phylogenetic data can help in better understanding of the structural diversity of antimicrobial peptides.