3. Materials and Methods
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3.1. Recombinant DNA Techniques for Cloning and DNA Analysis

3.1.1. Preparation of Competent Cells

*Escherichia coli* DH5α cells were subcultured in Luria Bertani (LB) medium (50 ml) from an overnight grown culture and incubated at 37°C till the $A_{600}$ reached 0.4. Cells were harvested by centrifugation at 2,200 X g for 10 min at 4°C. The pellet was resuspended in 20 ml of ice-cold 100 mM CaCl$_2$ and incubated on ice for 30 min. Cells were collected by centrifugation at 1,500 X g for 20 min at 4°C and resuspended gently in 1.5 ml of ice-cold 100 mM CaCl$_2$. To this cell suspension equal volume of 40% glycerol was added to bring the final concentration to 20%. Cell suspension (0.1 ml) was aliquoted into eppendorf tubes and stored at −80°C.

3.1.2. Polymerase Chain Reaction

Rapid amplification of the DNA fragments was done using 2 units of Taq DNA polymerase, 100 μM dNTPs and 10 picomoles of each primer. All the PCR reactions included a denaturation, an annealing and an extension step. The temperature of denaturation was 94°C, and of extension was 72°C but that of annealing varied with the Tm of the primers used. The time for denaturation and annealing was usually 30 sec, but that of extension varied depending on the size of the fragment to be amplified. The reaction was carried out for 35 cycles and the reaction product was electrophoresed on 1% agarose gel to check for the amplification. For performing colony PCR, a few cells picked from each of the isolated colonies were resuspended in sterile water and boiled for 5 min. The lysed cells were centrifuged at 12,000 X g for 5 min and the supernatant was used as the DNA template for PCR reaction. All the primers used for PCRs were obtained from IDT.

3.1.3. Purification of DNA Fragments from Agarose Gel

Restriction enzyme digested plasmid or PCR amplified products were electrophoresed on agarose gel in TAE buffer (40 mM Tris-acetate; 1 mM EDTA, pH 8.0). The desired fragment was identified using standard molecular weight marker (1 kb ladder from MBI Fermentas or 100 bp ladder from RBC) and purified using the QIAquick gel
extraction column (Qiagen), following manufacturer's instructions. The DNA fragment was excised from the agarose gel and collected in an eppendorf tube. Three volumes of solubilization buffer (Buffer QG) were added to 1 volume of gel and the gel slice was dissolved by heating at 55°C for 10 min. The mixture was loaded onto QIAquick spin column and centrifuged for 1 min. The flow-through was discarded and the bound DNA was washed with wash buffer (Buffer PE). The bound DNA fragment was eluted with 50 μl of elution buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). All the buffers used were supplied with QIAquick gel extraction kit.

3.1.4. Ligation and Transformation

Fragments amplified by PCR were cloned in pGEMT-easy vector (Promega) carrying 3'-T overhangs. The pGEMT-easy ligation reaction was carried out in water bath set at 4°C for overnight in the cold room and other ligations were carried out at 16°C for overnight. After stipulated incubation, the ligation mix was added to 100 μl of competent cells, mixed gently and incubated on ice for 30 min. Cells were subjected to heat shock at 42°C for 90 sec followed by addition of 900 μl of LB medium to the tube and incubated further at 37°C for 1 h with slow shaking for revival of cells. The revived cells were centrifuged at 750 X g for 5 min. After discarding 900 μl of LB medium, the cells were resuspended in the remaining 100 μl and plated on Luria agar plate containing the suitable antibiotic (depending on the vector used). If the vector allowed blue/white selection, 100 μl of 100 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 20 μl of 50 mg/ml X-Gal (5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside) was spread over the surface of agar plate before plating. The plates were incubated overnight at 37°C. In order to subclone an insert from one vector to another, either suitable restriction sites present in the multiple cloning site (MCS) of both the vectors were chosen or the restriction sites were included in the primer itself. Transformed cells containing recombinant plasmid were identified by performing colony PCR.

3.1.5. Isolation of Plasmid DNA

Plasmid DNA was isolated by alkaline lysis method as described in Sambrook et al. (1989). Bacterial cells containing the desired clone were grown overnight at 37°C in LB medium containing suitable antibiotic depending upon the vector and the host strain used. The cells were harvested by centrifugation at 5,000 X g for 5 min at room temperature. The
pellet was resuspended in 200 µl of solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0 and 50 mM glucose) and subsequently 400 µl of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and mixed by inversion. To the lysed cells, 300 µl of ice-cold solution III (7.5 M ammonium acetate) was added, mixed vigorously by inversion and incubated on ice for 20 min. The mixture was centrifuged at 13,400 X g for 15 min at 4°C. Supernatant (650 µl) was collected in a fresh tube and centrifuged again at 13,400 X g for 5 min at room temperature to remove any bacterial debris. Again, supernatant (550 µl) was collected in a fresh tube, to which 450 µl of isopropanol was added and mixed by inversion. The mixture was incubated at room temperature for 5 min and centrifuged at 13,400 X g to pellet the DNA. The pellet was washed with 70% ethanol and then air-dried. The pellet of nucleic acid was then dissolved in Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 50 µg/ml RNase A and incubated at 37°C for 30 min.

3.1.6. DNA Sequencing

The sequencing of the insert cloned in pGEMT-easy vector was carried out by Sanger’s dideoxy termination method (Sanger et al., 1977) with the help of T7 sequencing kit (Amersham). Vector specific or gene specific primers were used for sequencing depending upon the requirement. Briefly, two microgram of the plasmid DNA was denatured by heating (65°C) and using alkali (NaOH) and the primer was annealed to the template DNA by quick annealing method. The sequencing reaction was carried out in the presence of 35S-dATP at 21°C and then terminated as described in the protocol. The samples were boiled for 5 min and cooled on ice before loading. The labeled fragments were separated on a 5% polyacrylamide gel containing 8 M urea. Electrophoresis was carried out at constant power (40 watts) using 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0) and the temperature of the gel was maintained at 50°C. Three loadings were done to resolve maximum nucleotide sequence. After completion of the run, the gel was transferred to whatmann 3MM paper, dried and autoradiographed. The nucleotide sequence was read manually and analyzed by computer using MacVector version 7.0 software.

3.1.7. Spectrophotometric Estimation of Nucleic Acids

Quantity and purity of nucleic acids in solution was determined by measuring the absorbance at 260 and 280 nm. Concentration of DNA was calculated by taking A260 = 50
µg/ml for DNA and 40 µg/ml for RNA. The purity of nucleic acid solutions was checked by taking the A$_{260}$/A$_{280}$ ratio.

3.2. Polyacrylamide Gel Electrophoresis (PAGE) of Proteins

Polyacrylamide Gel Electrophoresis (PAGE) was performed according to the protocol of Laemmli (1970). Gels were prepared and electrophoresed in the presence of 0.1% SDS (denaturing). The composition of the separating and stacking gel mixtures are given in the following Table. Protein samples were prepared by adding 4X SDS sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 4% β-mercaptoethanol, 0.01% bromophenol blue). Samples were boiled for 5 min, centrifuged at 13,400 X g and loaded on the gel. Gels were electrophoresed using Tris-glycine (25 mM Tris-Cl, 250 mM glycine and 0.1% SDS) as running buffer, at 100 V till the proteins were stacked properly and thereafter gels were electrophoresed at a constant voltage of 120 V. Gels were stained with Coomassie Brilliant Blue (CBB) (0.05% Coomassie blue R-250, 25% isopropanol and 10% acetic acid) and destained with 10% acetic acid as described by Laemmli (1970).

Table2: Composition for 15% resolving and 5% stacking gel:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Resolving Gel (ml)</th>
<th>Stacking Gel (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide Stock Solution (30%)</td>
<td>2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>4X Resolving Gel buffer</td>
<td>1.25</td>
<td>-</td>
</tr>
<tr>
<td>(1.5 M Tris-HCl, pH 8.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4X Stacking Gel buffer</td>
<td>-</td>
<td>0.75</td>
</tr>
<tr>
<td>(0.5 M Tris-HCl, pH 6.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1.1</td>
<td>1.68</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>Ammonium persulphate (10%)</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005</td>
<td>0.004</td>
</tr>
</tbody>
</table>

3.2.1. Western Blotting

Western blotting was done according to the method described by Towbin et al. (1979). Mini Trans-blot Electrophoretic Cell (Bio-Rad) was used to transfer the proteins from
the gel to the nitrocellulose membrane (Hybond-C, Amersham). The apparatus for electroblotting was assembled according to the manufacturer's instructions. For western analysis, the proteins were electrophoresed in SDS-PAGE along with prestained marker (Broad range molecular weight; Bio-Rad) and then transferred to the nitrocellulose membrane electrophoretically in transfer buffer (39 mM glycine, 48 mM Tris base, and 20% methanol) at a constant current of 100 mA for 2 h at 4°C. The membrane was rinsed briefly in Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) and then incubated in blocking solution (3% Bovine Serum Albumin (BSA) in PBS) for 1 h with gentle shaking at room temperature. The membrane was washed twice for 10 min each with PBS. The washing was followed by 1 h incubation with primary antibodies [anti-PGRP antibody (1:5,000) or anti-His antibodies (1:10,000) (Sigma) in PBS containing 0.5% BSA] at room temperature with gentle shaking. Thereafter, the blots were washed twice with PBS for 10 min each. After washing, blots were transferred to alkaline-phosphatase conjugated secondary antibody (Sigma) solution [goat anti-rabbit (1:5,000) or goat anti-mouse (1:10,000) in PBS containing 0.5% BSA] and further incubated for 1 h. The blots were washed thrice with PBST (PBS containing 0.05% Tween 20) for 10 min each and twice with PBS for 10 min each. The protein-antibody complex was developed with alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.5) containing p-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Bio-Rad). The reaction was stopped by rinsing the blot with distilled water.

3.3. Insect Rearing

The culture of *Spodoptera litura* was obtained from Entomology department of Indian Agricultural Research Institute (IARI). A continuous colony of *S. litura* was developed in insect rearing facility of our laboratory having a controlled environment. The conditions were 25 ± 1°C temperature, 70% relative humidity with a photoperiod of 12:12 h (light: dark). The larvae were reared on castor leaves up to second instar and from third instar onwards on a semisynthetic diet having following composition.

3.3.1. Composition of the Semisynthetic Diet (for 880 g):

Part I: 110 g bengal gram powder, 20 g yeast powder, 10 g casein, 2 g methyl-p-hydroxy benzoate, 0.5 g sorbic acid, 1 ml formaldehyde and 420 ml distilled water
Part II: 2.6 g ascorbic acid, 0.115 g cholesterol, 0.1 g streptomycin sulfate, 1 g multivitamin mixture, 0.6 g vitamin E

Part III: 12 g bacto-agar and 300 ml distilled water

Agar was dissolved in 300 ml of boiling water and the gel was mixed thoroughly and cooked for 2 to 3 min. It was then poured into the dough of part I and mixed continuously in a blender. Ingredients of part II were added and blended again until an even consistency was obtained. The slurry of the diet was poured into petri dishes (14 cm diameter) and allowed to cool at room temperature and stored at 4°C for ripening and till further use. Pieces of the diet were offered to larvae in small petri dishes (5.5 cm diameter).

3.3.2. Challenging the Insect Larvae

*E. coli* K12 or *Micrococcus luteus* was subcultured from overnight grown culture and grown up to an absorbance of 0.5-0.6. The cells from 1 ml culture were harvested, washed with Ringer's solution (150 mM NaCl, 5 mM KCl, 3 mM CaCl₂ and 1mM NaH₂CO₃) and resuspended in 1 ml of Ringer's solution. Five microlitre of *E. coli* or *M. luteus* or both were injected into the hemolymph of *S. litura* larvae by inserting the needle into the proleg using microinjector (KPS 210, KD scientific, Newhope, PA). Same amount of Ringer's solution was injected as a control. The tissues (either fat body or hemocytes) were harvested at different time points after challenge and stored at −70°C in Trizol for isolating RNA.

3.4. Cloning of Peptidoglycan Recognition Protein

3.4.1. Isolation of Total RNA

Total RNA was isolated from *S. litura* fat body tissue using Trizol from Invitrogen. Tissues frozen in Trizol reagent (50 mg tissue/0.5 ml Trizol) were ground in an eppendorf tube using pellet pestle. To remove the debris and fat globules, the ground samples were centrifuged at 12,000 X g for 10 min at 4°C. The supernatant was brought to room temperature and mixed with 100 μl of chloroform and incubated at room temperature for 2-3 min. The mixture was then centrifuged at 12,000 X g for 15 min at 4°C. The aqueous phase was removed carefully and incubated with 250 μl of isopropanol at room temperature for 10 min. The mixture was centrifuged at 12,000 X g for 10 min at 4°C to pellet the RNA. The RNA
pellet was washed with 75% ethanol by centrifuging at 7,500 X g for 5 min, air-dried and dissolved in DEPC-water by heating at 60°C.

3.4.2. Isolation of mRNA

Total RNA was mixed with 3M KCl in such a way to bring the concentration of KCl to 500 mM. 1.0 μl of biotinylated oligo dT was added to the total RNA and incubated at room temperature for 15 min with intermittent shaking to allow mRNA to bind to biotinylated oligo dT. In the meantime, 25 μl of streptavidin magnetic beads were equilibrated with 500 mM KCl by mixing with 500 mM KCl and eluting with magnet few times. After 15 min total RNA was mixed with magnetic beads and incubated at room temperature for 5 minutes allowing the binding of biotinylated oligo (dT) to streptavidin magnetic beads. The mixture was eluted with magnet retaining mRNA and the flow through containing rRNA was discarded. The beads were washed with 500 mM KCl twice and 250 mM KCl once. DEPC-water was added to the beads and incubated at 65°C for 3 min. The beads were added to the magnet and the mRNA was eluted at 65°C.

3.4.3. cDNA Synthesis

Complementary DNA (cDNA) was synthesized using oligo (dT) primer with mRNA as template and reverse transcriptase (Superscript II, Life Technologies) as per manufacturer's instructions. Briefly, mRNA was added to 15 pmoles of oligo dT primer and initial denaturation was performed at 65°C for 5-10 min and the mixture was snap-cooled in ice. Reverse transcription was performed at 42°C for 50 min in the presence of RNase inhibitor and 5mM DTT and the reaction was stopped by incubating the mixture at 70°C for 15 min. RNA in DNA-RNA hybrid was digested with RNaseH by incubating at 37°C for 20 min.

3.4.4. Designing Degenerate Primers and Cloning Partial Sequence of pgrp Gene

The sequences of PGRP protein from Bombyx mori, Manduca sexta and Trichoplusia ni were aligned and the primers were designed from the conserved regions. The alignment and the position of the degenerate primers are shown in the figure 8. The conserved amino acid sequences were reverse translated and the degenerate bases were included at the appropriate positions.
Figure 8: Alignment showing the position of degenerate primers. The positions of degenerate forward primers are shown in solid arrows and that of degenerate reverse primers are shown in dashed arrows.

The degenerate primers used were

1. PGRP DegF1: 5' - YTB GTS RTS RTH CAR CAC - 3'
2. PGRP DegF2: 5' - TKT AYG ARG GMK CBG GNT GG - 3'
3. PGRP DegR1: 5' - GTA BAR YTT YCK BCC RGG - 3'
4. PGRP DegR2: 5' - SAR CTG BCG RTG VSC MAC - 3'
5. PGRP DegR3: 5' - TAM AYY TTN CCR TTR CCD CC - 3'

The PCR reaction was carried out using fat body cDNA as template, 15 picomoles each of forward and reverse primers along with 100 μM of each dNTP, 1X PCR buffer and 2.5 U Taq DNA polymerase (Qiagen) in a 25 μl reaction. The primer combinations used were PGRP DegF1 X PGRP DegR1 or PGRP DegR2 or PGRP DegR3 and PGRP DegF2 X PGRP DegR1 or PGRP DegR2. The primer combination of PGRP DegF1 X PGRP DegR3 yielded a product of 170 bp at 50°C annealing temperature. The PCR amplified DNA product
was resolved by electrophoresis on a 1% agarose gel. The amplified DNA fragment was purified from the gel, cloned into pGEMT-easy vector and sequenced as described above.

3.4.5. 5' and 3' Rapid Amplification of cDNA Ends (RACE) of pgrp Gene

To get the 3' and 5' ends of PGRP 3' RACE and 5' RACE were performed by using the respective kits (Invitrogen) according to the manufacturer's instructions.

3' RACE was performed as given in the figure 9.

**Figure 9: Schematic Representation of 3' RACE**

cDNA was prepared using challenged fat body mRNA as a template, oligo (dT) Adapter Primer and SuperScript II RT enzyme. RNA in the RNA:DNA hybrid was degraded by using RNase H. 3' RACE first PCR was performed using cDNA as a template and PGRP_3RACE.2 (5'-AGA TCG TGA GGA ATA TAC A-3') as a forward primer and Abridged Universal Amplification Primer (AUAP) from the 3' RACE kit as a reverse primer. Conditions for the PCR included a hot start of 1 min followed by 35 cycles each of 94°C for 30 sec, 47°C for 30 sec and 72°C for 30 sec. The first PCR product was reamplified with gene specific nested primer, PGRP_3RACE.1 (5'-AAT CAT ATG GAT AAC TTG AA-3') as a forward primer and AUAP as a reverse primer. The PCR conditions were as follows: 35 cycles each of 94°C
for 30 sec, 45°C for 30 sec and 72°C for 30 sec. The 500 bp fragment obtained from this PCR was eluted from the agarose gel and ligated into pGEMT-easy vector. The ligation mixture was transformed into E. coli DH5α cells and the clones were checked by colony PCR. Plasmids were isolated from five positive clones and sequenced using T7 sequencing kit (Amersham Biosciences).

5' RACE was performed as given in the figure 10.

Figure 10: Schematic Representation of 5' RACE

First strand cDNA was synthesized using challenged fat body mRNA as a template, gene specific primer, PGRP_5RACE.1 (5' - GCC GCC AAT AAT GAA CGA C - 3') and SuperScript II RT enzyme. RNA was degraded using RNase mix (RNase H and RNase T1) and the cDNA was purified by passing through the QiAgen gel extraction column. The cDNA was dC tailed using terminal deoxytransferase (TdT) and dCTP. dC-tailed cDNA was PCR amplified using Abridged Amplification Primer (AAP) as a forward primer and gene specific primer, PGRP_5RACE.2 (5' - CGA CGA TCC AAT ATC CCA CA - 3') as a reverse primer. Conditions for the PCR reaction included a hot start of 1 min followed by 35 cycles each of
94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. The PCR product was run on 1% agarose gel and 300 bp PCR product was eluted from the agarose gel and ligated into pGEMT-easy vector. The ligation mixture was transformed into E. coli DH5α cells and the clones were checked by colony PCR. Plasmids were isolated from five positive clones and sequenced using T7 sequencing kit (Amersham Bioscience).

3.4.6. Cloning of Full Length pgrp Gene

Two specific oligonucleotides, PGRP_for1 (5' - ATG AAG TTA TTT TTT AAC ATT TTC GC - 3') and PGRP_rev1 (5' - TTA GTT CTT CAA TGA GCT GAC ATC - 3') corresponding to sequence of 5' and 3' ends of the PGRP cDNA were synthesized. These primers were used to amplify the complete ORF of PGRP using the challenged fat body cDNA as template. Conditions for the PCR included a hot start of 1 min followed by 35 cycles each of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The PCR amplified DNA product was electrophoresed on a 1% agarose gel, purified from the gel, cloned into pGEMT-easy vector and sequenced as described above. Five independent clones containing the full length gene were sequenced.

3.5. Cloning of Antimicrobial Peptide Genes

The antimicrobial peptides were cloned from the cDNA of immune challenged fat body of S. litura. Primers used for cloning antimicrobial peptide genes from S. litura were

S.I._Cecropin_for 5'- ATC TTT TTC TTT GTG TTC GCG - 3'
S.I._Cecropin_revA 5'- TTA TCC AAG AGC CTT GGC TTG T - 3'
Spodoptericin_for 5'- ATG GGT GTA AAG GTA ATA AA T G - 3'
Spodoptericin_rev 5'- TTA AAT GCA GCT ACATGT GTG- 3'
Cobatoxin_for 5'- ATG AAA CTT ATA CTG TTT GTC G - 3'
Cobatoxin_rev 5'- TTA ACT GTA GCA ACG ACA GG - 3'
Gallerimycin_for 5'- ATG AAG GCT TGC GTG GTT CTC - 3'
Gallerimycin_rev 5'- CTA TCG CAG ACA TTG GCA TCC - 3'

The primers of cecropin were designed based on the sequence of S. litura cecropin sequence given in Choi et al. (2000). The primers for other antimicrobial peptide genes were
designed based on the *Spodoptera frugiperda* gene sequences appeared in Volkoff *et al.* (2003). Annealing for cecropin A, cobatoxin and spodoptericin was 54°C and for gallerimycin was 58°C. The amplified product was electrophoresed on 1% agarose gel, eluted from the gel and cloned into pGEMT-easy vector and the positive clones were identified by colony PCR. The plasmids from five to ten positive clones were sequenced and the identity was confirmed.

3.6. Challenging of *S.litura* Hemocytes Cell Line

One million *S. litura* hemocytes cells (from NIV, Pune) were seeded in a 6-well plate and stimulated with 3 μM hydroxy ecdysone (Sigma) for 72 h and then challenged with actively growing 10 μl of *E. coli* and *M. luteus* mixture. The cells were harvested in 500 μl Trizol and stored at -70°C after different time points.

3.6.1. One-step RT-PCR

RNA was isolated from the *S. litura* hemocytes cell line and treated with DNase and checked for DNA contamination. If the RNA was found DNA free, then equal quantity of RNA (around 100ng) was used for One-step RT-PCR (Qiagen). The primers used for β-actin were β-actin_For 5’- CAG ATC ATG TTT GAG ACC TTC AAC- 3’ and β-actin_Rev 5’ - GA/C/TC CAT CTC C/TGG CTC GAA A/GTC - 3’ and for PGRP were PGRP _RT.F2 5’- AAC AAC AAT GGG ACG GGT TA - 3’ and PGRP _RT.R2 5’- GCT TGA GTT GGG GTG TCA TT - 3’. The primers used for cloning antimicrobial peptides were used for one-step RT-PCR. The reaction components and the conditions used were according to the manufacturer’s instructions. RT-PCR was run for 30 cycles.

3.7. RNA interference Experiments

3.7.1. Double-stranded RNA Preparation

The recombinant pGEMT-easy vector carrying pgrp gene was used as a template for PCR amplification with universe primer (5’-GTA AAA CGA CGG CCA GT-3’) and reverse primer (5’-GGA AAC AGC TAT GAC CAT G-3’) (universe primer sequence lies upstream of the T7 promoter sequence in pGEMT-easy vector and that of reverse primer lies downstream of the SP6 promoter). The amplified fragment was purified from the gel as described above and used as a template for *in vitro* transcription using both T7 and SP6 RNA polymerase. The
*in vitro* transcription was carried out using 5 μg of DNA template, 2 mM rNTPs, 60 U of RNA polymerase, 20 U of RNase inhibitor and 1X transcription buffer in a reaction volume of 100 μl. The reaction was carried out at 37°C for 2 h and stopped by incubating at -20°C for 15 min. Two single-stranded RNAs (ss-RNAs) were treated with DNase (1 U/μg of DNA used in the transcription reaction). For making ds-RNA, the two ss-RNAs were mixed and incubated at 65°C for 30 min followed by slow cooling to room temperature. The annealed ds-RNA was purified by phenol:chloroform extraction and precipitated with ice-cold ethanol in the presence of 0.3 M sodium acetate by incubating overnight at -20°C. The ds-RNA was pelleted by centrifuging at 13,400 X g at 4°C for 30 min and washed in 75% ethanol made in DEPC-water. The pellet was dissolved in DEPC-water and checked on 0.8% agarose gel.

### 3.7.2. Injection of ds-RNA into Insect Larvae

Five microgram of PGRP-dsRNA was injected into the hemolymph of sixth instar second day larvae. Same volume of DEPC-water was injected as a negative control. 10 h after injection with ds-RNA, the larvae were challenged with 5 μl of the mixture of actively growing *E. coli* K12 and *M. luteus*. 12 h after challenge the larvae were dissected and fat body tissues and hemocytes were recovered and stored in Trizol at -70°C till further use. Usually, the tissues from 2 larvae were pooled and stored in 500 μl of Trizol.

### 3.7.3. Northern Hybridization

Equal concentration of total RNA (3.0 μg from fat body tissues and 15 μg from hemocytes tissues) was electrophoresed on 1.2% formaldehyde denaturing agarose gel according to Sambrook *et al.* (1989). The gel was electrophoresed in 1X MOPS (20 mM MOPS, pH 7.0, 2 mM sodium acetate and 1 mM EDTA (pH 8.0) buffer at 70 V at room temperature. After completion of the run, the gel was rinsed in DEPC-treated water for 4 times 5 min each and with 10X SSC (1.5 M NaCl and 0.15 M sodium acetate, pH 7.0) for 10 min. RNA was transferred to nylon membrane (Amersham Hybond-N) by capillary transfer in 10X SSC for 16 h. The RNA blot was rinsed with 2X SSC and UV-crosslinked. The blot was stained with methylene blue and destained with excess of distilled water. Pre-hybridization was carried out in pre-hybridization buffer (5X SSC, 5% dextran sulfate, 50 mM sodium hydrogen phosphate (pH 7.2), 5X Denhardt’s solution, 2.5 mM EDTA (pH 8.0), and 0.4% SDS) at 65°C for 3-5 h in a hybridization oven. During pre-hybridization, salmon sperm DNA
was added to the buffer at a concentration of 100 μg/ml for blocking. The probe was made by using Nick translation kit (Amersham BioSciences). Respective gene fragments after PCR amplification and purification was used for making probe. The probe was boiled for 5 min; snap cooled and added to the pre-hybridization buffer and hybridization was carried out overnight either at 65°C (for β-actin and PGRP) or at 60°C (for antimicrobial peptides). The blot was washed twice with each of the following buffers 2X SSC containing 0.1% SDS at room temperature for 10 min, 0.5X SSC containing 0.1% SDS at 65°C for 15 min. Finally the blot was rinsed with 2X SSC at room temperature for 10 min to remove SDS. The blot was then exposed to the phospho imager screen in the phosphoimager cassette for few hours and the screen was scanned using Typhoon scanner (Amersham). The image was analyzed using Image Quant software.

3.8. Expression of Peptidoglycan Recognition Protein in E. coli

To subclone pgrp gene into prokaryotic expression vectors, the gene was amplified with PGRP_for2 (5’- GGA TCC ATG AAG TTA TTT TTT AAC - 3’) containing BamHI recognition site and PGRP_rev2 (5’- GTC GAC TTA GTT CTT CAA TGA GC - 3’) containing SalI recognition site. Conditions for the PCR included a hot start of 1 min followed by 35 cycles each of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec and a final extension at 72°C for 10 min. The PCR product was ligated into pGEMT-easy vector. The positive clones were identified by colony PCR. The plasmid was isolated from a positive clone and the full length pgrp gene was excised from pGEMT-easy vector by digesting it with BamHI and SalI restriction enzymes. The excised fragment was ligated to BamHI and SalI digested pQE 30 vector at 16°C for overnight and transformed into competent cells of E. coli M15 strain, the expression host for pQE series of vectors. Plating was done on agar plates containing 100 μg/ml of ampicillin and 20 μg/ml of kanamycin and incubated overnight at 37°C. The positive clones were identified by carrying out colony PCR, using gene specific primers. The excised full length PGRP gene was also cloned into the pET 28a, pET 29a, pET 32a, pET 32b, pGEX 4T1 and pGEX 4T3 vectors. The recombinant pET 28a and pET 29a vector was transformed into E. coli BL21(DE3) and Rosetta cells. The recombinant pET 32a vector was transformed into E. coli BL21(DE3) and Rosetta cells. The recombinant pET 32b vector was transformed into E. coli BL21(DE3), AD494(DE3), C43(DE3);pLysS and Origami cells. The recombinant pGEX 4T1 and pGEX 4T3 were transformed into E. coli BL21(DE3) cells. The antibiotic used for selecting pET 28a, pET 29a vectors, Origami and AD494 (DE3) cells was kanamycin @
50 µg/ml and for pET 32a, pET 32b, pGEX 4T1 and pGEX 4T3 was ampicillin @ 100µg/ml. The antibiotic used for selecting Rosetta was chloramphenicol 34 µg/ml. The positive clones were identified by colony PCR and restriction digestion. These clones were also sequenced to verify the correct ORF and the sequence.

The transformed clone harboring the recombinant plasmid was grown in LB medium containing appropriate antibiotic, to an A600 of 0.6 and induction was carried out with 1 mM IPTG for 3 h. One of the cultures was left uninduced and served as a negative control. The cells were harvested by centrifugation and resuspended in 1X PBS buffer containing 1 mM PMSF (phenylmethylsulfonyl fluoride). The cells were lysed by sonicating the cell suspension for 3 X 30 sec at an output of 50 W using Sonics Vibra cell Sonicator and the cell lysate was centrifuged at 12,000 x g for 20 min at 4°C. Both the pellet and the supernatant were resolved on 15% SDS-PAGE and checked for the expression of the recombinant protein.

3.9. Expression of PGRP in Sf21 Cells

3.9.1. Maintenance of Sf21 Cells

The IPLB-Sf21 cell line, originally derived from Spodoptera frugiperda (Vaughn et al., 1977) were grown and maintained at 27°C in TNM-FH medium (BD-Biosciences) (Grace's basic medium with yeastolate, lactalbumin hydrolysate and L-glutamine supplemented with 10% FBS and 50 µg/ml gentamicin). Cells were grown as a monolayer in tissue culture flasks (Nunc). Cell viability was determined by the trypan blue exclusion method.

3.9.2. Expression of PGRP protein using Bac-to-Bac Baculovirus Expression System

Expression of PGRP protein using Bac-to-Bac Baculovirus expression system is depicted in the figure 11 and mainly involves the following steps:

1. Cloning of pgrp gene into donor plasmid

2. Transformation of MAX Efficiency DH10Bac competent E. coli cells with the pFastBac construct to generate a recombinant bacmid

3. Transfection of the recombinant bacmid DNA into the Sf21 insect cell line to generate a recombinant baculovirus
4. Amplification and titration of baculoviral stock, and use of this stock to infect insect cells and to express recombinant protein

![Diagram of Bac-to-Bac Baculovirus Expression System]

Figure 11: Schematic Representation of Bac-to-Bac Baculovirus Expression System

3.9.2.1. Cloning of *pgrp* Gene into Baculovirus Donor Vector pFASTBac1

To clone *pgrp* gene into the baculovirus donor vector, pFastBac HTB (Invitrogen), the gene was amplified with PGRP_for2 (5′- GGA TCC ATG AAG TTA TTT TTT AAC - 3′) containing BamHI recognition site and PGRP_rev2 (5′- GTC GAC TTA GTT CTT CAA TGA GC - 3′) containing SalI recognition site; and to clone into the vector, pFastBac 1 (Invitrogen), the gene was amplified with gene specific primers, PGRP_FaBac_For (5′- GAA TTC AAA ATG GAG TTA TTT TTT AAC ATT T - 3′) containing EcoRI site and Kozak consensus sequence, and PGRP_FaBac+His_Rev (5′- AAG CTT TTA GTG ATG ATG ATG ATG GTT CTT CAA TGA GCT GAC ATC - 3′) containing HindIII site and 6X-His tag and cloned into pGEMT-easy vector. The gene was excised from pGEMT-easy vector by digesting with BamHI and SalI and cloned into BamHI/SalI-digested pFastBac HTB vector or excised by digesting with EcoRI and HindIII and cloned into EcoRI/HindIII-digested pFastBac 1 vector following standard recombinant DNA techniques (Sambrook *et al.*, 1989). Cloning was
confirmed by restriction enzyme digestion of the recombinant donor vector. The recombinant donor vector carrying the \textit{pgrp} gene was termed pFastBac HTB-\textit{pgrp} or pFastBac-\textit{pgrp} as the case may be. Plasmid DNA of this clone was prepared by midi prep using Qiagen kit.

\textbf{3.9.2.2. Transformation of DH10Bac Competent Cells}

\textit{E. coli} DH10Bac cells contain baculovirus shuttle vector (bacmid), bMON14272 (136 Kb), containing kanamycin resistance marker, a low-copy number mini-F replicon and a segment of DNA encoding the LacZ\textsubscript{\alpha} peptide into which the attachment site for the bacterial transposon, Tn7 (mini-\textit{attTn7}) has been inserted, a helper plasmid, pMON7124 (13.2 Kb) encoding transposase and providing the transposition function in \textit{trans} and conferring tetracycline resistance. DH10Bac competent cells were transformed either with the recombinant pFastBac 1-\textit{pgrp} vector or with pFastBac- HTB-\textit{pgrp} following standard heat shock method, except for the revival of the cells after heat shock was for 4 h instead of 1 h, giving enough time for the transposition to take place. After revival, the cells were serially diluted and plated on LB agar plate containing 50 \(\mu\)g/ml kanamycin, 7 \(\mu\)g/ml gentamicin, 10 \(\mu\)g/ml tetracycline, 100 \(\mu\)g/ml Bluo-gal and 40 \(\mu\)g/ml IPTG and incubated at 37\(^\circ\)C for 48 h. The large and white positive colonies were selected and bacmid DNA were isolated from them using midi prep method (Qiagen). The bacmid DNA was subjected to PCR using M13 forward (-40) (5'- GTT TTC CCA GTC ACG AC - 3') and M13 reverse (5' - CAG GAA ACA GCT ATG AC - 3') primers and the PCR conditions involved initial denaturation for 2 minutes at 94\(^\circ\)C followed by 35 cycles of denaturation at 94\(^\circ\)C for 30 sec, annealing at 55\(^\circ\)C for 30 sec and extension at 72\(^\circ\)C for 3 min and a final extension for 10 min at 72\(^\circ\)C. The positive clones yielded 2,430 bp + 576 bp PCR product. The positive bacmid DNA was used for transfecting Sf21 insect cells.

\textbf{3.9.2.3. Transfection of Sf21 Cell Line}

One million Sf21 cells were transfected by following the instructions given in the Invitrogen manual. Briefly, 1-2 \(\mu\)g of recombinant bacmid DNA was diluted in 100 \(\mu\)l of SFM (Serum Free Medium) and 6 \(\mu\)l of cellfectin reagent was also diluted in 100 \(\mu\)l of SFM. Both the diluted bacmid DNA and cellfectin was mixed and incubated at room temperature for 30 min to allow the formation of DNA:lipid complex. 0.8 ml of SFM was added after 30 min to the mixture and added to the adhered Sf21 cells in the 6-well plate dropwise and incubated at 27\(^\circ\)C for 5 h. A mixture lacking cellfectin, containing only recombinant bacmid DNA, served as
a negative control. After 5 h, DNA:lipid complex was removed and 2 ml of TNM-FH was added to the cells and incubated at 27°C for 72 h. After 72 h, the supernatant containing recombinant baculovirus BV-PGRP, P1 viral stock was harvested and stored at 4°C protected from light.

3.9.2.4. Amplification of Viral Stock

The P1 viral stock obtained after transfection was further amplified by infecting Sf21 cells in an MOI (multiplicity of infection) ranging from 0.05 to 0.1, assuming the MOI of P1 viral stock to be 1 X 10^6 to 1 X 10^7 pfu/ml (on an average 5 X 10^6 pfu/ml). 2 X 10^6 Sf21 cells were seeded per well of a 6-well plate and allowed to attach for an hour at room temperature. After an hour, the appropriate amount of P1 viral stock was added to each well and left at 27°C for 72 h. The medium containing virus was collected and spun at 500 x g for 5 min to remove cells and large debris. The supernatant was transferred to a new tube and stored at 4°C for regular use and at -70°C for long term storage, protected from light. This P2 viral stock was again amplified to get P3 viral stock.

3.9.2.5. Plaque Assay

Plaque assay was performed to obtain a pure recombinant clone of individual plaque and to determine the MOI of viral stock. The transfection supernatant or a viral stock was serially diluted to give final dilutions of 10^-1 to 10^-6 using TNM-FH medium as the diluent. One million cells were seeded in each well of 6-well tissue culture plate. The cells were infected with 100 μl of each of the diluted virus, incubated at room temperature for 1 h with gentle shaking in between. During this incubation, sterile 2% SeaPlaque agarose was melted and cooled to 37°C. The virus inoculum was removed from the cells after the stipulated incubation period. Equal volume of warm TNM-FH medium was added to the 2% agarose and mixed properly. The infected cell monolayer was overlaid with 1.5 ml of this 1% agarose solution. Once the agarose overlay had set, 1.5 ml of TNM-FH medium was added to each well. The plate was incubated at 27°C for 4-5 days.

Each well was overlaid with 0.03% of neutral red. Viral plaques were identified as clear (unstained) areas and confirmed by observing under a microscope. To get a pure recombinant virus, the plaques were lifted by pushing the 1 ml pipette tip through the agarose overlay into the plaque and gently sucking the agarose plug into the tip. The plug was
released into the microcentrifuge tube containing 0.5 ml of TNM-FH medium, vortexed and stored at 4°C for overnight to allow viruses to diffuse out of the agarose. MOI of viral stock was calculated by counting the well isolated plaques and considering the dilution factor, number of cells and the amount of medium used in the well.

3.9.2.6. Optimization of PGRP Expression in Sf21 Cells

A time course study was carried out to determine the level of expression of the recombinant PGRP protein as a function of time. One million Sf21 cells were infected at a MOI of 5 and were harvested 24 h, 48 h, 72 h and 96 h postinfection. After washing twice with 1X PBS, the cells were lysed and resolved on 15% SDS-PAGE and analyzed by western blotting as described above. Western analysis was carried out by using anti-His antibodies (1:10,000). The expression was also checked by infecting the cells at different MOI, 1, 2, 5, 10 and 20 and analyzed the cells after 72 h of infection.

3.10. Solubilization and Purification of PGRP Protein

The infected cells harvested from 10 X T 75 tissue culture flasks were washed with 10 ml of 1X PBS and resuspended in 1X PBS containing 8 M urea, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1mM PMSF. The resuspended cells were left on the nutator at room temperature for an hour to solubilize the protein and centrifuged at 13,400 X g for 20 min at 4°C. The supernatant was mixed with the Ni-NTA resin pre equilibrated with buffer A (1X PBS + 8 M urea) and left on the nutator at room temperature for 2 h. It was then loaded on a column and washed with 2-3 bed volumes of buffer B (buffer A + 20 mM imidazole). A linear gradient was run from 8 M urea to 0 M urea using buffer B and buffer C (1X PBS + 20 mM imidazole). Then the column was washed with 2-3 bed volumes of buffer D (buffer C + 10% glycerol). Finally, the elution was carried out with buffer E (buffer D + 300 mM imidazole) in 10 X 500 μl aliquots. The fractions containing PGRP protein were pooled and dialyzed against buffer D using 10 kDa dialysis bag for overnight at 4°C and concentrated using 10-kDa cut-off centricon (Millipore) by following manufacturer’s instructions.

3.11. Protein Concentration Determination

Protein concentration of samples was determined by dye-binding method of Bradford (1976) using BSA (Sigma) as the standard.
3.12. Production of Polyclonal Antibodies

The purified PGRP protein was injected into five female Balb/c mice to raise antibody. Antiserum was raised by immunization of each Balb/c mouse by administrating the emulsion made from 20 µg of PGRP and Freund's complete adjuvant (Sigma). The mice were boosted two times with emulsion made from 20 µg of PGRP protein and Freund's incomplete adjuvant (Sigma). The mice were bled 7 days after first and second boost and the blood was incubated at 37°C for an hour followed by incubation at 4°C for overnight. The serum was separated from the blood cells by centrifuging at 10,000 rpm for 15 min at 4°C and stored at -20°C in aliquots. The reactivity of the mice serum was assessed by western blotting.

3.13. Immunofluorescence Localization

In a 12-well tissue culture plate, 0.5 X 10^6 Sf21 cells were seeded onto a sterile microscope coverslip (18 mm diameter). The cells were then infected with recombinant virus at MOI of 2 for 36 h at 27°C. After stipulated incubation, the infected cells were washed three times with 1X PBS. The cells in each well were fixed with 1 ml of 2% formaldehyde in PBS. The fixed cells were washed three times with 1X PBS and blocked with 3% BSA in PBS at room temperature for 1 h. Following three washes with 1X PBS, the cells were incubated with anti-PGRP polyclonal serum (1:5,000) in PBS containing 0.5% BSA at room temperature for 1 h. After incubation, cells were washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:50) in PBS containing 0.5% BSA for 1 h at room temperature. Finally cells were washed with 1X PBS and mounted using vectashield hard set mounting medium. The cells were observed under fluorescence microscope and photographed.


The antibacterial assays were carried out as described in Selsted et al. (1984) with minor modifications. Briefly, M. luteus and Enterococcus casseliflavus were grown to an A_600 of 0.5 and 0.3, respectively and serially diluted to 10^6 and 10^4 fold, respectively. 50 µl of diluted culture was incubated with 2.5 µg of purified PGRP at 37°C for 30 min and plated on LB agar plate and incubated overnight at 37°C. In parallel, PBS treated bacterial cultures
were also plated as a control. After overnight incubation colonies were counted and the number of colony forming units per ml was calculated for all the treatments.

3.15. Binding Assay of PGRP with Different Classes of Microbes

Gram-negative bacteria like E. coli and Enterobacter aerogenes. Photorhabdus luminescens, Xenorhabdus nematophila, Gram-positive bacteria like E. casseliflavus, M. luteus, Staphylococcus aureus, Bacillus subtilis and Bacillus thuringiensis were grown till A₆₀₀ reached 0.5-0.6. The cells were pelleted and resuspended in 100 μl of 1X PBS. To this suspension, 2 μg of purified PGRP protein was added and left at room temperature for 30 min. It was mixed gently once a while. Then the mixture was centrifuged at 8,000 rpm for 5 min. The pellet was washed twice in 1 ml of 1X PBS and finally resuspended in 100 μl of buffer. Equal volumes of supernatant and pellet was loaded on a 15% SDS-PAGE and blotted to a nitrocellulose membrane. Western analysis was done with anti-PGRP primary antibody and alkaline phosphatase conjugated anti mouse secondary antibody and developed with NBT-BCIP.

3.16. Binding Assay of PGRP with Different Classes of Microbial Ligands

Peptidoglycan of E. coli K12 (Invivogen), M. luteus (Invivogen) and S. aureus (Invivogen) and chitin (Sigma) and β-1,3 glucan (Sigma) were resuspended in 10 mM Tris-maleate buffer pH 6.5. All the peptidoglycans were made to a final concentration of 1mg/ml and chitin and β-1,3 glucan were made to a final concentration of 10 mg/ml. 100 μl of each component was mixed with 2 μg of PGRP protein and incubated at 4°C for 45 min mixing once a while. After the stipulated incubation time, it was pelleted down and washed twice with 1 ml of 10 mM Tris-maleate buffer, pH 6.5 and resuspended in 100 μl of 10 mM Tris-maleate buffer, pH 6.5. Equal volumes of supernatant and pellet were loaded on a 15% SDS-PAGE gel and western analysis was done as described in 3.15.

3.17. Ligand recognition specificity of PGRP using ELISA

The sonicated peptidoglycan of S. aureus (6.25 μg in 25μl) was coated per well in the 96-well ELISA plate (Nunc) by passing warm air over it. The unbound sites in the microplate wells were blocked with 3% BSA for 2 h and washed twice with 1X PBS. 300 ng of purified PGRP either alone or after incubating with various concentrations of ligands (incubated with N-acetylglucosamine (Sigma), muramic acid (Sigma), Muramyl dipeptide...
(MDP: N-Acetylmuramyl-L-Alanyl-D-Isoglutamine from Invivogen), Glucosaminyl muramyl dipeptide (GMDP: N-Acetyl-D-glucosaminyl-(β1,4)-N-acetylmuramyl-L-alanyl-D-isoglutamine from Calbiochem), Tri-DAP (L-Alanyl-D-γ-glutamyl-meso-diaminopimelic acid from Invivogen) and Muramyl Tri-DAP (MTP: N-acetyl-muramyl-L-Alanyl-D-γ-glutamyl-meso-diaminopimelic acid from Invivogen) for 2 h at room temperature) was used to bind to the peptidoglycan coated on the well for 1 h. The unbound protein was washed with 1X PBS twice and the wells were incubated with 50 μl of anti-PGRP antibodies (1:2500) for 1 h. After washing twice with 1X PBST and once with 1X PBS, 50 μl of HRP-conjugated anti-mouse antibody (Bangalore Genei, India) (1:1000) was added to each well for 1 h. Then the wells were washed with 1X PBST twice and once with 1X PBS. The colour development was carried out by incubating wells with 50 μl of 1X TMB (3,3',5,5'- tetramethyl benzidine from Bangalore Genei, India) for 15 min and the reaction was stopped with 50 μl of 1 M H2SO4 per well. The yellow colour developed was quantified at 450 nm using ELISA reader.

3.18. Site Directed mutations

A series of site directed mutations were performed using Quickchange site directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The residues mutated were His51 to Ala, Cys57 to Ala, Cys63 to Lys, Thr165 to Lys and Ser167 to Ala. The pFastBac1-pgrp plasmid was used as a template. Few clones from each mutation were sequenced to confirm the change in the residue. Further, protein expression and purification were done as described for wild type protein.

3.19. Real time RT-PCR analysis for sippo1 gene

E. coli K-12 cells were subcultured from overnight grown culture and grown for 4 h. These cells were pelleted and resuspended in sterile Ringer’s solution to a concentration of 2 X 10⁹ cells/ml. Sixth instar second day larvae were injected with this inoculum (5 μl/insect) by piercing through the proleg using a microinjector (KPS 210, KD scientific, Newhope, PA) with Hamilton syringe. Five microlitre plain Ringer’s solution was injected as a control. Hemolymph from different stages of insect larvae, Ringer’s solution injected, and E. coli injected larvae after 6 and 18 h was collected in pre-chilled anticoagulant buffer and spun at 700 X g for 5 min at 4°C. The hemocyte pellet was homogenized in Trizol and RNA was isolated according to the manufacturer’s instructions (Invitrogen). Contaminating genomic DNA was removed by treating with RQ1 DNase (Promega) at 37°C. Real time analysis was done using Quantitect
SYBR Green RT-PCR kit (Qiagen) in the iCycler iQ system (Bio-Rad). The sequences of the primer pair used for amplifying β-actin gene were forward: 5'- CAG ATC ATG TTT GAG ACC TTC AAC - 3' and reverse: 5'- GA/C/TC CAT CTC C/TTG CTC GAA A/GTC-30. The primer pair for amplifying slppo1 gene is forward: 5'- AAC CAA CGT AGT TGG GTT GA - 3' and reverse: 5'- AAC TGC TGA TGG TGG GTT TCT A - 3'. Each 20 μl reaction contained 2 μl RNA template (100-500 ng), 10 μl of 2X QuantiTeet SYBR Green RT-PCR Master Mix, 10 picomoles of each appropriate primer, 0.2 μl of QuantiTect RT Enzyme Mix, and DEPC treated water to make up the volume. Real time cycler conditions used were as follows: preliminary reverse transcription at 48°C for 30 min, an initial activation step at 95°C for 15 min and 40 cycles of denaturation at 95°C, annealing at 52°C, and extension at 72°C for 30 sec each followed by gradual temperature increase from 50°C to 94°C at the rate of 1°C/10 sec to enable melt-curve data acquisition. Each experiment was carried out in duplicate. The threshold cycles (CT) were obtained for slppo1 and β-actin transcripts from each experiment. Difference between CT of the reference gene, β-actin, and the gene of interest, slppo1 (ΔCT) was determined and the relative abundance of the transcript was calculated following comparative CT method using the formula $2^{Δ^ΔCT}$ (Pfall, 2001).

3.20. Expression of S. litura PPO1 in E. coli

S. litura prophenoloxidase gene (slppo1) was cloned in-frame in pQE30 as a BamHI and KpnI fragment and the resultant recombinant plasmid was transformed into E. coli M15 cells. E. coli M15:pQE30+slppo1 cells were grown until $A_{600}$ reached 0.5, following which it was induced with 1 mM IPTG and allowed to grow further for 3 h. The cells were pelleted, resuspended in 50 mM sodium phosphate buffer (pH 7.5) containing 8 M urea, and mixed in a nutator at room temperature for 1 h and centrifuged at 12,000 X g for 15 min at room temperature and the supernatant fraction containing the solubilized protein was recovered. The protein was purified by binding the supernatant to Nickel-NTA in a nutator for 1 h. The slurry was loaded on a Bio-Rad column and extensively washed with buffer (50 mM sodium phosphate, pH 7.5). The protein was eluted in buffer containing 250 mM imidazole and the salt was removed by dialyzing against 50 mM sodium phosphate, pH 7.5, with three changes. Western analysis was done using anti-PPO antibodies (raised against M. sexta PPO and used 1:2000 dilution) as a primary antibody and AP-conjugated anti rabbit IgG as secondary antibody and finally developed with NBT-BCIP substrate.
3.21. Phenoloxidase Enzyme Assay

To examine whether the *E. coli* expressed protein was catalytically active, it was subjected to a phenoloxidase assay as described by Hall *et al.* (2005), with minor modifications. One ml of reaction mixture containing 0.5, 1.0, 1.5, and 2.0 μg protein with 0.02% CPC (cetylperidinium chloride), was incubated at room temperature for 10 min and finally 2 mM substrate (4-methyl pyrocatechol) was added and the increase in absorbance at 528 nm was read in spectrophotometer (Amersham Biosciences). PPO inhibition assay was performed using the specific inhibitor phenyl thio-urea (PTU) at 5 mM concentration in the reaction mixture containing 1 μg of the enzyme protein. Specific activity of PPO was calculated using extinction coefficient of the substrate at 528 nm as 4230 M⁻¹cm⁻¹ (Nagai and Kawabata, 2000).