CHAPTER-3
CLONING, EXPRESSION AND CHARACTERISATION OF CHPV PROTEINS
3.1 Introduction

Genomic RNA of Chandipura virus (CHPV) encodes five proteins; nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large protein (L) [8]. The functional and structural information of the CHPV proteins has been extended from the research literature on the prototype virus of the genus i.e., Vesicular Stomatitis Virus (VSV). Nucleocapsid protein encapsidates the viral RNA genome and forms the Nucleocapsid-RNA (N-RNA) thus protecting the RNA from degradation during various stages of viral life cycle. N-RNA is present in association with viral RNA dependent RNA polymerase (RdRp) which is composed of L protein (the catalytic subunit) and phosphorylated P protein (the transcriptional activator). The complex of L, P and N proteins along with RNA is known as ribonucleoprotein (RNP) particle [7]. P protein is the regulatory protein responsible for the switch between transcription and replication. During virus assembly, the M protein interacts with the ribonucleoprotein particle and condenses it into a tightly packed helix, giving the virus its characteristic bullet shaped morphology. The M protein associated RNP is surrounded by a lipoprotein envelope that contains trimers of G protein spiking out from the virion surface [48]. G protein is a single pass type I transmembrane protein, which has a short cytoplasmic tail, a transmembrane domain and an antigenic ectodomain [135, 136]. It is the only spike protein of CHPV that assists virus adsorption, assembly and budding [53]. Viral entry, a critical step in the life cycle of the virus, is mediated by G protein.

In addition to its structural role, Matrix (M) protein balances virus transcription [49, 50] and replication by condensation of nucleocapsid in tightly coiled helix [137]. It has been shown to be responsible for directing the virus budding from infected host cells [13, 14]. It inhibits the mRNA nuclear export by directly interacting with host factors, hence shutting off cellular transcription [52, 138]. M has also been known to induce cell rounding and apoptosis in infected cells, hence responsible for the cytopathic effects [51].

In view of the fact that VSV and CHPV have differences in their target hosts, lethality and phylogeny, it becomes essential to study these viral proteins from CHPV source itself and understand biological functioning. So far, only N and P proteins of CHPV have been purified and are being studied for their functional roles in CHPV life cycle. Recombinant G protein expression however, has been reported recently using baculovirus and Pichia Pastoris expression systems [59, 60] but its purification has never been reported using bacterial expression system. In the present study, four of the CHPV proteins (N, P, M and G) were
cloned and overexpressed in bacterial system. The proteins were analysed and optimized for solubilisation using different tags and changes in growth conditions and were successfully purified. CHPV M and G proteins have been purified for the first time through a bacterial system. As biology of CHPV remains less studied to date, the availability of clones/protocols for purified proteins can augment research in this direction. The expressed proteins can also find application in characterization of their function, structure, interactions and other biological studies. Interaction analysis using these proteins has been performed in subsequent chapters.
3.2 Materials and methods

3.2.1 Bacterial expression vectors

The *Escherichia coli* (*E. coli*) expression vector systems employed in the present study included a pair of vectors, namely pLTAExp20 and pCAKExp10 and a third vector system pGEX-4T3. Vector pGEX-4T3 (Novagen, USA) enabled the expression of CHPV viral genes fused with GST tag (Figure 3.1). Vectors pLTAExp20 (carry His tag) and pCAKExp10 (carry Strep tag) were kindly gifted by Dr. Amita Gupta, Department of Microbiology, University of Delhi South Campus, India (Figure 3.2). The characteristic features of above vectors are listed following in detail (Table 3.1).

**Table 3.1: Key features of the bacterial expression vectors**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Size</th>
<th>Origin of replication</th>
<th>Tag</th>
<th>Promoter</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX-4T3</td>
<td>4.9 kb</td>
<td>pBR322</td>
<td>GST (N terminal)</td>
<td>Lac</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>pLTAExp20</td>
<td>5.6 kb</td>
<td>ColE1</td>
<td>His (N terminal)</td>
<td>Tet</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>pCAK Exp10</td>
<td>5.5 kb</td>
<td>ColA</td>
<td>Strep (N terminal)</td>
<td>Ara</td>
<td>Kanamycin</td>
</tr>
</tbody>
</table>

![Vector Map of pGEX-4T3 expression vector](image)

**Figure 3.1: Vector Map of pGEX-4T3 expression vector**
3.2.2 Primer design

Oligonucleotide primers were designed to amplify the Nucleocapsid (N), Phosphoprotein (P), Matrix (M) and Glycoprotein (G) genes of Chandipura virus. The sense and antisense primers were designed to incorporate suitable restriction enzyme sites chosen for the vectors, pGEX-4T3, pLTA and pCAK. Additional nucleotides 5' to the restriction site were also included to facilitate enzyme binding (Table 3.2).

CHPV genes cloned in pUC and pET vectors (Table 3.3) and their sequencing results (unpublished data) were kindly received from Prof. Dhrubajyoti Chattopadhyay, Kolkatta, India [20, 45].

![Figure 3.2: Vector Maps of (a) pLTA and (b) pCAK expression vectors](image-url)
### Table 3.2: Primers used to amplify CHPV genes for cloning in bacterial expression vectors

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N F Smal I (pGEX-4T3)</td>
<td>5’ ATCCCCCAGGGGCAGTTCTCAAGTTCTGTACATTTC 3’</td>
</tr>
<tr>
<td>2</td>
<td>N R Xho I (pGEX-4T3)</td>
<td>5’ CATCTCGAGTCATGCAAAAGAGTTCTGGC 3’</td>
</tr>
<tr>
<td>3</td>
<td>P F Smal I (pGEX-4T3)</td>
<td>5’ AGAGCCCAGGCGAAGACTCGCAACTGTATCAA 3’</td>
</tr>
<tr>
<td>4</td>
<td>P R Xho I (pGEX-4T3)</td>
<td>5’ TGCCCTCGAGTCAAATGTGAATGGTCAAG 3’</td>
</tr>
<tr>
<td>5</td>
<td>M F EcoRI (pGEX-4T3)</td>
<td>5’ CTCCAAATTCCAAACGTCTGAAGTTTATAG 3’</td>
</tr>
<tr>
<td>6</td>
<td>M R Not I (pGEX-4T3)</td>
<td>5’ TGCCCGCCCGCTCAATGACTCTTAGTTACAGC 3’</td>
</tr>
<tr>
<td>7</td>
<td>G F EcoRI (pGEX-4T3)</td>
<td>5’ CAGGAATTCTATTTGATAGCATTTCCAG 3’</td>
</tr>
<tr>
<td>8</td>
<td>G R Not I (pGEX-4T3)</td>
<td>5’ CACCGCGCCGCTCATACTCTGCTGCTATGT 3’</td>
</tr>
<tr>
<td>9</td>
<td>N F (pLTA &amp; pCAK)</td>
<td>5’ CGGCAGCAGTTCTCAAGTTCTGTAA 3’</td>
</tr>
<tr>
<td>10</td>
<td>N R (pLTA &amp; pCAK)</td>
<td>5’ CTCCACCTCATGCAAAGTTTCTGG 3’</td>
</tr>
<tr>
<td>11</td>
<td>P F (pLTA &amp; pCAK)</td>
<td>5’ CGGCAGCAGACTCGCAACTGTACAA 3’</td>
</tr>
<tr>
<td>12</td>
<td>P R (pLTA &amp; pCAK)</td>
<td>5’ CTCCACCTCAATTGAACTGGCTCAAG 3’</td>
</tr>
<tr>
<td>13</td>
<td>M F (pLTA &amp; pCAK)</td>
<td>5’ CGGCAGCAGCTTCTGAAGTTTATAG 3’</td>
</tr>
<tr>
<td>14</td>
<td>M R (pLTA &amp; pCAK)</td>
<td>5’ CTCCACCTCATGCAAAGTTTCTGG 3’</td>
</tr>
<tr>
<td>15</td>
<td>G F (pLTA &amp; pCAK)</td>
<td>5’ CGGCAGCTATTTGAGTATAGCATTTCCAG 3’</td>
</tr>
<tr>
<td>16</td>
<td>G R (pLTA &amp; pCAK)</td>
<td>5’ CTCCACCTCATGCAAAGTTTCTGG 3’</td>
</tr>
</tbody>
</table>

Gene specific primers used for PCR amplification of Chandipura virus N, M, P and G genes (F- Forward primer and R- Reverse primer). The names of the restriction enzymes are in italics and their recognition sequences in bold. Both pLTA and pCAK vectors require same set of forward and reverse primers and are part of strategy which does not need restriction digestion of the PCR product (explained in section 3.2.6.1).

### Table 3.3: Details of the CHPV gene clones used as templates for PCR

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Plasmid</th>
<th>Plasmid Size</th>
<th>Gene</th>
<th>Gene Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pET 33b</td>
<td>5.3 kb</td>
<td>N</td>
<td>1.2 kb</td>
</tr>
<tr>
<td>2</td>
<td>pET3a</td>
<td>4.6 kb</td>
<td>P</td>
<td>0.8 kb</td>
</tr>
<tr>
<td>3</td>
<td>pUC19</td>
<td>2.6 kb</td>
<td>M</td>
<td>0.7 kb</td>
</tr>
<tr>
<td>4</td>
<td>pUC19</td>
<td>2.6 kb</td>
<td>G</td>
<td>1.5 kb</td>
</tr>
</tbody>
</table>
3.2.3 Gene amplification by polymerase chain reaction

The pUC and pET vectors harbouring CHPV viral genes (Table 3.2) were used as templates for PCR amplification. All PCR amplifications were performed in sterile 0.2 ml microcentrifuge tubes in Programmable Thermal Cycler (DNA Engine, BioRad, USA). Amplification reactions were carried out using Taq DNA polymerase as well as Pfu, a high fidelity DNA polymerase. An initial test reaction (analytical reaction) of 20 µl was set up using 0.5 pmol, each of forward and reverse primer, 0.25 mM deoxyribonucleotides (dNTPs), 1.5 U of Taq and 1 ng of template DNA. Once PCR product sizes were confirmed by electrophoresis, preparative PCR reactions were performed in a final volume of 100 µl using 1 U of Pfu polymerase in addition to 6 U of Taq DNA polymerase. The reaction was carried out for 30 cycles, each cycle consisting of denaturation at 95 ºC for 45 seconds, annealing at 55 ºC for 45 seconds and extension at 72 ºC for 1 minute for each gene except G gene which was amplified by keeping extension time of two minutes.

3.2.4 Purification of plasmids and PCR products

The PCR products were purified using the Qiagen PCR purification kit (Qiagen, Germany) as per the manufacturer’s guidelines. The DNA samples to be purified were mixed with five times the volume of binding solution and loaded into spin columns containing a silica-based membrane that adsorbs double stranded DNA. The spin column was placed into 2 ml collection tube and centrifuged at 13000 rpm for 1 minute. The flow through was discarded and the spin column was washed twice with membrane wash solution (700 µl followed by 400 µl). The column was centrifuged at 13000 rpm for 1 minute during each washing step. The spin column was transferred to fresh 2 ml collection tube and 50 µl of nuclease free water was added directly to the centre of the membrane. The column with the collection tube was incubated at room temperature for 1 minute followed by centrifugation at 13000 rpm for 1 minute to finally elute the DNA and the purified product was stored at -20 ºC.

3.2.5 Agarose gel electrophoresis

DNA was electrophoresed on 1.2% (w/v) agarose gel [with 0.5µg/ml ethidium bromide (EtBr)] in 1X TAE buffer (Appendix A). DNA loading dye (6X) (Appendix A) was added to the samples to make the final concentration to 1X. The sample was loaded into the well and each gel included a well containing 500 ng of the DNA Ladder (1 kb or 100 bp
ladder; Fermentas, USA). Gels were electrophoresed in 1X TAE at 100V till the dye front reached 3/4\textsuperscript{th} of the gel length. Following electrophoresis, DNA bands were visualised using BioRad Quantity One gel documentation system.

3.2.6 Cloning of PCR amplified genes in bacterial expression vectors

3.2.6.1 Restriction enzyme digestion

The DNA fragments (~1 µg; Vector and PCR product) were treated with 5 U of each restriction enzyme (mentioned in primer details; Table 3.3; Fast Digest Enzymes; Fermentas). The digestion was carried out in a reaction volume of 100 µl in the presence of 10 µl of 10X fast digest buffer at 37 °C for 2 hours. For generating pLTA and pCAK based recombinants, PCR purified amplicon was not digested rather treated with T4 DNA polymerase (15 U) and dTTP (20 mM) for 1 hour at 15 °C to generate Bsa I compatible ends [139]. The vector (pLTA/pCAK) was digested with Bsa I enzyme and a stuffer fragment was released. The plasmid backbone was purified by extracting the required DNA band (larger fragments, i.e., 3.9 kb for pLTA and 3.8 kb for pCAK vector) from the agarose gel using Gel Extraction Kit (Sigma Aldrich, USA).

3.2.6.2 Ligation reaction

For ligations, vector:insert in the molar ratio of 1:5 was optimized. Ligation reactions comprised of 50 ng vector (25 ng for pLTA/pCAK vectors), DNA insert (according to molar ratio), 2 µl of 10X Ligation Buffer and 3.0 U T4 DNA ligase (Fermentas) in a final reaction volume of 20 µl. The mixtures were incubated at 22 °C for 1 hour followed by 4 °C again for 1 hour and used for bacterial transformation.

3.2.6.3 Preparation of competent bacterial cells

DH5\textalpha/BL21 (E. coli) cells were streaked onto LB agar plates from frozen stocks (-80 °C) and incubated at 37 °C overnight. A single colony was inoculated into 4 ml of LB broth and incubated at 37 °C overnight at 200 rpm. The following day, 2 ml of this culture was transferred to a 1L flask containing 200 ml of LB broth, incubated at 37 °C for ~3 hours at 200 rpm for log phase until the OD\textsubscript{600} was 0.4-0.5. The cultures were transferred to pre-chilled 50 ml tubes and centrifuged at 6000 rpm for 7 minutes at 4 °C. The supernatant was removed and the bacterial pellet was resuspended and pooled in 100 ml of ice-cold 50 mM CaCl\textsubscript{2}
followed by incubation for 60 minutes on ice. The suspension was centrifuged and the bacterial pellet was resuspended in 20 ml of ice-cold 50 mM CaCl₂. The bacterial cells were quickly centrifuged at 6000 rpm for 7 minutes at 4 °C and resuspended in 2 ml of ice-cold 50 mM CaCl₂ containing 20% glycerol. Bacterial cells were now stored in 100 µl aliquots at -80 °C. The transformation efficiency of the competent cells was checked with the help of a control plasmid DNA of known concentration.

3.2.6.4 Bacterial transformation

Ligation mixture was transformed into competent DH5α (for cloning purposes) or BL21 (for protein expression) E. coli cells. Transformation was carried out by gently mixing 5 µl of the ligation reaction with 40 µl of competent cells (thawed on ice). The transformation reaction was then placed on ice for 20 minutes followed by heat shock at 42 °C for 60 seconds. After heat shocked the tube was immediately chilled in ice for 2 minutes. This was followed by the addition of LB medium (300 µl) and the incubation of the transformation mixture at 37 °C for 1 hour with shaking at 200 rpm. The mixture (100 µl) was plated onto LB agar plates containing respective antibiotic required for the vector [ampicillin (100 µg/ml) for pGEX 4T3/pLTA and kanamycin (30 µg/ml) for pCAK]. The plate was incubated overnight at 37 °C.

3.2.6.5 Preparation of bacterial glycerol stocks

Bacterial cultures containing recombinant plasmid were cryo-preserved by inoculating a single colony into 4 ml of LB broth with appropriate antibiotic. The cultures were allowed to grow overnight at 37 °C with shaking at 200 rpm. Glycerol was added to a final concentration of 20% to the cultures and 1 ml aliquots in cryovials were stored at -80 °C.

3.2.6.6 Plasmid DNA isolation using alkaline lysis method

Transformed bacterial colonies were individually inoculated in 4 ml of LB broth with appropriate antibiotic and incubated overnight at 37 °C with shaking at 200 rpm. The bacterial cells from 3 ml of overnight culture were harvested at 6000 rpm for 1 minute at 4 °C and the supernatant was removed completely. The bacterial pellets were resuspended by vortexing in 200 µl of ice-cold resuspension solution (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose, 100 µg/ml RNase A and 4 mg/ml lysozyme). The cells were then lysed by the addition of 400 µl of freshly prepared lysis solution (1% SDS in 0.2 N NaOH) and inversions
of tubes 5-6 times. Following cell lysis, 300 µl of ice-cold potassium acetate solution (3 M potassium acetate pH 5.5; Appendix A) was added to neutralize the reaction and to precipitate bacterial chromosomal DNA and proteins. The cells were mixed with the solution by inversions, stored on ice for 5 minutes and centrifuged at 12000 rpm for 15 minutes at 4 °C. The supernatant (~800 µl) was carefully removed and transferred to fresh 1.5 ml microcentrifuge tube. The plasmid DNA was precipitated by addition of 500 µl of isopropanol followed by inversions and incubation in ice for 5 minutes. This was followed by centrifugation at 12000 rpm for 10 minutes at 4 °C. The supernatant was discarded; DNA pellet was washed with 70% ethanol and air dried at room temperature. Plasmid DNA was resuspended in 50 µl of nuclease free water and stored at -20 °C.

3.2.6.7 Plasmid DNA isolation using QIAGEN kit

Miniprep plasmid DNA preparations were carried out using the QIAGEN MINI prep kit. The procedure is based on the alkaline lysis method, but uses a support column to bind and purify plasmid DNA. The bacterial pellet (from 3 ml culture) obtained after harvesting the cultures was treated with solution I, II and III (designated as P1, P2 and N3, respectively in the kit). The sample was centrifuged at 12000 rpm for 10 minutes at 4 °C and the supernatant were then applied to kit spin columns. The plasmid DNA in supernatant gets bound to the silica matrix of the column. Wash buffer (700 µl) was applied to the spin column and it was centrifuged for 1 minute at 12000 rpm at room temperature. Nuclease free water (50 µl) was added to the column and incubated for 1 minute at room temperature. The plasmid was eluted by centrifugation at 12000 rpm for 1 minute. The size of the plasmid DNA and the presence of insert in the recombinant vector were confirmed by restriction enzyme digestion. The plasmid DNA preparation by this method was used for transformation and cloning purposes.

3.2.7 Expression and solubilisation of bacterial fusion proteins

The recombinant clones for N, P, M and G genes of CHPV were transformed in BL21 strain of E. coli to carry out the protein expression studies. Transformed cells were grown in 30 ml LB medium supplemented with appropriate antibiotic. The cultures were induced with IPTG (for pGEX-4T3), anhydrotetracycline (for pLTA) and arabinose (for pCAK) as applicable. The conditions for induction were standardized by varying temperature (16 °C, 25 °C and 37 °C), induction time (16 hours, 4 hours and 2 hours) and inducer concentrations (0.2% and 0.5% for arabinose, 20 ng, 50 ng and 100 ng/ml for anhydrotetracycline). The
induced cells were harvested by centrifugation at 6000 rpm for 6 minutes at 4 °C. The induced cell pellets were lysed using IBA Lysis buffer (IBA-GmbH, Germany; 1 mM EDTA, Tween 20 and avidin) according to the manufacturer’s guidelines. Protease inhibitor cocktail (Sigma Aldrich), lysozyme (1 µg/ml) and nucleases [DNase (20 ng/ml), RNase (6 ng/ml)] were also added along with the lysis buffer and all the steps of cell lysis were carried out at 4 °C. After lysis, the supernatant (soluble fraction) and the pellet (insoluble fractions) were collected by centrifugation at 13000 rpm for 20 minutes at 4 °C and analysed by 10% SDS-PAGE to check for the presence of the protein of interest.

3.2.7.1 Solubilisation of Glycoprotein using sarkosyl

The CHPV G protein, observed in the pellet (insoluble fraction), after cell lysis (from 30 ml culture) was treated with sarkosyl, a detergent used to solubilise the protein from inclusion bodies. Solubilisation buffer (400 µl) containing 50 mM Tris, 5 mM ZnCl₂, 300 mM NaCl, 10 mM β-mercaptoethanol and 10% sarkosyl was mixed with the pellet obtained after cell lysis and incubated overnight at 16 °C [140]. The solubilised protein was recovered as supernatant by centrifugation at 13000 rpm for 10 minutes. Supernatant was diluted to 1% final concentration of sarkosyl. The sample was further dialysed in 1X PBS (Phosphate Buffer Saline) buffer for 20 hours with four changes of buffer at 4 °C. Cellulose acetate dialysis tubing with cut off of 12 kDa (Sigma Aldrich) was used for dialysis. Introduction of dialysis step was a modification of the protocol described by Tao and co-workers [140]. The dialysed fraction was then checked for the presence of G protein by SDS-PAGE.

3.2.8 Purification of CHPV M and G proteins as GST fusions

The soluble/dialysed fraction of M and G protein as GST fusion (500 µl) was loaded on 300 µl of Glutathione sepharose beads (Clontech, USA) and incubated at 4 °C for 2 hours with gentle agitation. The samples were centrifuged at 2100 rpm for 5 minutes at 4 °C to remove the unbound protein fraction [labeled flowthrough (FT)]. The beads were washed by suspending them in 1.5 ml of 1X PBS buffer followed by centrifugation. The washing step was repeated three times. After washing, the beads were incubated with 300 µl of elution buffer [10 mM reduced glutathione (Sigma Aldrich) in 50 mM Tris-HCl, pH 9.0] at room temperature for 15 minutes for elution of the target protein. The sample in supernatant was collected by centrifugation at 2100 rpm for 5 minutes at 4 °C and labeled as E1. The elution buffer was added second time to the beads and eluate collected (E2). The eluted samples (E1
and E2) along with the flow through (FT) were analysed by electrophoresis on 10% SDS-polyacrylamide gel.

3.2.9 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was carried out as described by Laemmli (1970) [141]. Protein samples were resuspended in sample buffer (20 mM Tris-HCl, pH 6.8, 2% sodium dodecylsulfate, 5% β-mercaptoethanol, 2.5% glycerol and 2.5% bromophenol blue) and denatured at 100 °C for 5 minutes. The Mini Protean polyacrylamide gel electrophoresis apparatus (BioRad, USA) was assembled according to the manufacturer’s instructions and 10% resolving gel was pipetted into the gel spaces. Gel was left to polymerise at room temperature for 30-45 minutes. After polymerization of the resolving gel, 5% stacking gel solution was poured over. Well comb was inserted and the stacking gel was left to polymerise at room temperature. Following gel polymerisation, comb was removed, wells were rinsed with distilled water and the apparatus was filled with 1X running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Unstained protein ladder; Fermentas (10-200 kDa range) was used to establish the apparent molecular weights of proteins resolved on the SDS polyacrylamide gel. The prestained protein ladder; Fermentas (10-170 kDa) was used for the gels to be used for western blotting. The protein samples in polyacrylamide gel were allowed to run at 100 V.

3.2.9.1 Coomassie brilliant blue staining

Polyacrylamide gel was stained by immersing the gel in Coomassie brilliant blue staining solution (0.25% Coomassie brilliant blue R250, 45% methanol, 10% acetic acid) for 15 minutes at room temperature with gentle agitation. The gel was destained in Coomassie de-staining solution (30% methanol, 10% acetic acid) thrice for 30 minutes with gentle agitation.

3.2.9.2 Western Transfer

Following polyacrylamide gel electrophoresis the apparatus was disassembled and the gel was placed in western transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Transfer cassettes were assembled with all its parts pre-wetted in transfer buffer. A scourer was placed on the black (negative) side of the cassette, followed by two pieces of Whatman 3 MM paper, the gel, a piece of polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, USA) two pieces of Whatman paper and another scourer. Cassettes were closed and placed in the transfer apparatus. Proteins were electroblotted onto PVDF membrane at 100
mA for 1 hour or at 30 mA overnight in transfer buffer.

3.2.9.3 Western Blotting

Following protein transfer to PVDF membrane, the apparatus was disassembled and the membrane was blocked with 5% BSA in 1X PBS, pH 7.4, for 1 hour to mask the non-specific sites. The membranes were then incubated with monoclonal mouse anti GST (1:10000; Sigma Aldrich, USA; for GST fusions) or monoclonal mouse anti His (1:3000; Sigma Aldrich; for His fusions) or monoclonal mouse anti Strep (1: 4000; IBA-GmBH, Germany; for Strep fusions) antibodies as applicable for 1 hour. This step was followed by washing the blot three times (10 minutes each) with 1X PBS containing 0.05% Tween-20 (PBS-T). Membranes were then incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody (1:2000, G Biosciences, India) for one hour. The blot was washed three times with PBS-T followed by three times with PBS. The immunoblots were visualised by the addition of 3, 3’diaminobenzidine (DAB; 0.05 %) as substrate for HRP and hydrogen peroxide (0.1%). The reaction resulted in the appearance of an insoluble brown product at the site of tagged proteins in the blot. All incubations for western blotting were carried out at room temperature on a horizontal shaker.
3.3 Results

3.3.1 Cloning of CHPV N, P, M and G genes in pGEX-4T3 expression plasmid

CHPV N, P, M and G genes were cloned in pGEX-4T3 vector (Section 3.2.1) to express, solubilise and purify encoded proteins as GST fusions. The N, P, M and G genes were PCR amplified with gene specific primers (Table 3.2) designed with suitable restriction sites for cloning in the vector, pGEX-4T3 using conditions described in section 3.2.3. PCR products of N (1200 bp), P (800 bp), M (690 bp) and G (1500 bp) genes were observed at expected sizes after electrophoresis on 1.2% agarose gel (Figure 3.3). The amplified and purified N and P PCR products were digested with Sma I and Xho I while M and G genes with EcoR I and Not I restriction enzymes. Vector pGEX-4T3 was digested with enzyme combinations corresponding to PCR products, purified and ligated. The ligated mixtures were transformed in competent E. coli DH5α cells and the transformants were screened by preparing the plasmid DNA from the recombinant colonies (that grew on LB selection media) using alkaline lysis method followed by their restriction enzyme digestion.

N gene cloning was confirmed by digestion with Sma I and Xho I, the combination used for cloning, to observe 1.2 kb band in agarose gel electrophoresis (Figure 3.4). P gene cloning was confirmed by Sma I digestion where size shift for digested recombinants was compared to control vector. The shift in the size corresponded to the size of P gene i.e., 800 bp (Figure 3.5). M and G gene cloning was confirmed with EcoR I and Not I (Figure 3.6 and 3.7) enzymes to release a fragment of 690 bp indicative of M gene and 1.5 kb for G gene. A control restriction digestion reaction containing non-recombinant vector was set up during each experiment (Lane C in Figures 3.4, 3.5, 3.6 and 3.7). Recombinants generated by cloning N, P, M and G genes in pGEX-4T3 vector were named as pGEX-N, pGEX-P, pGEX-M and pGEX-G respectively.
Figure 3.3: PCR amplification of N, P, M and G genes of CHPV using pGEX-4T3 primers
Amplified N, P, M and G PCR products with gene specific primers for cloning in pGEX-4T3 vector. All the products were observed at their expected sizes. Lanes 1, 2, 3 and 4 represent amplified N gene (1200 bp), P (800 bp), M (690 bp) and G genes (1500 bp), respectively. L2 is 1 kb DNA ladder (Sigma Aldrich; Molecular sizes are indicated in kb).

Figure 3.4: Restriction digestion of pGEX-N using Sma I and Xho I
Miniprep DNAs were screened for recombinants by restriction enzyme digestion using Sma I and Xho I enzymes to observe the released fragment expected at 1.2 kb corresponding to N. N1-N3 (lanes 3-5) are the recombinant clones. L1 is 1 kb DNA ladder (Fermentas; Molecular sizes are indicated). C (lane 2) is the control vector digested with the same enzyme combination.
Figure 3.5: Restriction digestion of pGEX-P using Sma I

The minipreps prepared from the transformed colonies were screened for recombinants by Sma I restriction enzyme digestion to observe the shift in size of the recombinant clone. P2 (lane 2) is the positive clone. L2 is 1 kb DNA ladder (Sigma Aldrich; Molecular sizes are indicated). C (lane 3) is the control vector digested with Sma I.

Figure 3.6: Restriction digestion of pGEX-M using EcoR I and Not I

The recombinant minipreps of CHPV M gene in pGEX-4T3 vector were screened by restriction enzyme digestion using EcoR I and Not I to observe the released band expected at 690 bp for M. M1-M3 (lanes 2-4) represents positive clones. L1 is 1 kb DNA ladder (Fermentas; Molecular sizes in kb are indicated). C (lane 5) represents the control vector digestion.
3.3.2 Expression and solubilisation of GST-CHPV fusion proteins

The GST fusion clones of N, P, M and G genes, i.e., pGEX-N, pGEX-P, pGEX-M and pGEX-G were analysed for the expression and solubilisation profile of viral proteins. The recombinant plasmids were transformed in *E. coli* BL21 cells and induced with 1 mM IPTG (Isopropyl β-D-thiogalactopyranoside) at 37 ºC for 2 hours. Cells expressing the recombinant proteins were lysed, centrifuged and the presence of the proteins was checked in the soluble (supernatant) and insoluble (cell pellet) fractions. Analysis by SDS-PAGE and western blot using anti GST monoclonal antibodies revealed that the N, P and M proteins were partially soluble i.e., were detected in the supernatant as well as cell pellet fractions. Therefore the conditions such as induction temperature and induction time were standardized to increase the solubility of the proteins. Lower induction temperature (25 ºC) was tried as the tendency of the proteins to form aggregates decreases with the decrease in temperature. Accordingly, the time for induction was increased from 2 hours to 4 hours for induction at 25 ºC. The analysis revealed that at 37 ºC, only a small fraction of N, P and M proteins were found to be soluble. However at 25 ºC for 4 hours, the solubility increased for all three proteins i.e., proteins were present in more abundance in the supernatant after cell lysis. Figures 3.8 and 3.9 indicate the solubilisation profile using SDS-PAGE and Western blot analysis, respectively. The expressed proteins of N [Figure 3.8 (panel a) lane 8 and Figure 3.9 lane 2], P [Figure 3.8 (panel a) lane 3 and Figure 3.9 lane 7] and M [Figure 3.8 (panel c) lane 5 and Figure 3.9 lane 9] were obtained in the supernatant (sup) fractions. However G protein, despite variations in
conditions was found to be insoluble i.e., was present in the cell pellet fraction [Figure 3.8 (panel b) lane 5 and Figure 3.9 lane 3]). The proteins were observed at their expected sizes [GST-N; 73 kDa, GST-P; 59 kDa, GST-M; 52 kDa, GST-G; 95 kDa] after analysis on SDS-PAGE and western blot except P protein which was observed at 72 kDa. P protein of negative sense RNA viruses is known to exhibit aberrant mobility [142, 143] and was thus observed at a higher size than expected. The expression of P and G proteins was observed to be less as compared to N and M proteins at same induction conditions.

![Figure 3.8](image)

**Figure 3.8**: Solubilisation analysis of CHPV N, P, M and G proteins as GST fusions

BL21 cells harbouring pGEX-N, pGEX-P, pGEX-M and pGEX-G plasmids were induced with IPTG at 25 °C for 4 hours. Cell pellets were collected before (BI) and after induction (AI). After induction cells were harvested, lysed and centrifuged. The soluble (supernatant, sup) and insoluble (pellet) fractions collected by centrifugation were analysed by SDS-PAGE. Panel (a) shows the solubilisation analysis of N and P proteins. Lanes 1-4 represents BI, AI, sup and pellets fractions for P protein solubilisation analysis, while lanes 6-9 represent similar solubilisation analysis for N protein. Panel (b) shows the solubilisation analysis of G protein, while panel (c) represents analysis for M protein [Marker is the prestained Protein ladder (Fermentas; molecular sizes are indicated in kDa)].
Expressed and solubilised GST fusion CHPV proteins were analysed by western blotting. The cell pellets were harvested, lysed and centrifuged. The soluble (supernatant, Sup) and insoluble (pellet, Pl) fractions were analysed using anti-GST monoclonal antibodies. Various samples are represented as; Pl and Sup from N in lane 1 and lane 2 respectively; Pl and Sup from G in lane 3 and lane 4 respectively; Pl and Sup from P in lane 6 and lane 7 respectively, and Pl and Sup from M in lane 8 and lane 9 respectively. Lane 5 is the prestained protein ladder (Fermentas; molecular sizes are indicated in kDa).

3.3.3 Cloning of CHPV genes in His and Strep tagged bacterial expression vectors

G protein was observed to be present in the insoluble fraction after cell lysis in the solubilisation studies using GST tag. Owing to the requirement of soluble G protein in interaction studies and keeping in mind the importance of G protein purification using bacterial system (unreported for CHPV and for family rhabdoviridae), smaller tags such as 6X His and Strep were also used as fusion with G protein. His and Strep tags are used in affinity based protein purification and their utilization in protein interaction studies are well demonstrated [144, 145]. Furthermore, due to the requirement of soluble CHPV proteins (N, P, M and G) as fusions with at least two different tags for viral-viral protein interaction studies, the tags were selected for further studies. CHPV genes were therefore cloned in pLTA (5.6 kb; His tag) and pCAK (5.5 kb; Strep tag) expression vectors [139]. The vectors were digested with enzyme which released a stuffer sequence from the vector backbone (1.73 kb for pLTA vector and 1.75 kb for pCAK vector). The inserts were prepared by PCR amplification (Figure 3.10) with gene specific primers (Table 3.3) followed by dTTP and T4 DNA polymerase treatment resulting in compatible ends for cloning in BsaI digested vector. The inserts and vectors were ligated and the pLTA fusion constructs thus generated were named as pLTA-N, pLTA-P, pLTA-M and pLTA-G. The recombinants were screened by digestion with NcoI enzyme (cuts in the vector backbone) to observe the shift in the size of
recombinants compared to the control pLTA vector (containing stuffer region). Digested control vector was observed at higher size due to the presence of the stuffer sequence (1.73 kb) in comparison to recombinants. P, G, N and M recombinants were 0.9 kb, 0.2 kb, 0.5 kb and 1 kb shorter than the control vector (Figure 3.11 and 3.12). Sma I enzyme (cuts in the vector backbone) was used for the analysis of pCAK recombinants (Figure 3.13, 3.14 and 3.15). The higher size of the control vector in these confirmations was again due to the presence of the stuffer sequence (1.75 kb) in control pCAK vector. The pCAK-CHPV gene fusion constructs were named as pCAK-N, pCAK-P, pCAK-M and pCAK-G. Thus, all the four CHPV genes were also cloned as His and Strep tag fusions in pLTA and pCAK vectors, respectively.

![Image](image.png)

**Figure 3.10:** PCR amplification of N, P, M and G genes for cloning in pCAK and pLTA Vectors

N, P, M and G genes were amplified with specific primers for cloning in pCAK and pLTA vectors. The amplified genes were observed at expected sizes on 1.2% agarose gel. Lane 2 is N gene (1200 bp), lane 3 represents P gene (800 bp), lane 4 is M gene (690 bp) and lane 5 is G gene (1500 bp). L1 is 1 kb DNA ladder (Fermentas; Molecular sizes in kb are indicated).

![Image](image.png)

**Figure 3.11:** Restriction digestion of pLTA-P, pLTA-G and pLTA-N using Nco I

Recombinant clones of P, G and N genes in pLTA vector were screened by restriction enzyme digestion using Nco I. Lanes 1-5 represents the clones of P (P1-P5), Lanes 6-8 indicate the clones of G (G1-G3), and Lanes 9-11 are representative of the clones of N (N1-N3). L1 is 1 kb DNA ladder (Fermentas; Molecular sizes are indicated in kb). C (lane 12) is the control vector digested with the same enzyme.
Figure 3.12: Restriction digestion of pLTA-M using Nco I
Screening for recombinants of M-pLTA clones by restriction enzyme digestion with Nco I. Lanes 1-10 indicate clones of M (M1-M10). L1 is 1kb DNA ladder (Fermentas; Molecular sizes are indicated in kb). C (lane 11) is the control vector digested with Nco I.

Figure 3.13: Restriction digestion of pCAK-M using Sma I
Recombinants for M-pCAK clones were screened by restriction enzyme digestion using Sma I to observe the shift in size of the recombinant DNA relative to the control vector. Lanes 1, 2, 3 and 4 represents miniprep clones of M-pCAK (M1-M4). L1 is 1 kb DNA ladder (Fermentas; Molecular sizes are indicated in kb). C (lane 5) is the control vector digested with Sma I.
G-pCAK Miniprep DNAs were screened for recombinants by \textit{Sma} I restriction digestion. G1- G4 (lanes 1-4) are the miniprep clones. L1 is 1 kb DNA ladder (Fermentas; Molecular sizes are indicated in kb). C (lane 5) is the control vector digested with \textit{Sma} I.

Recombinants of pCAK-P and pCAK-N were screened by restriction enzyme digestion using \textit{Sma} I. Lane 1 represents \textit{Sma} I digested miniprep of pCAK-P, whereas lanes 2-6 (N1-N5) are pCAK-N minipreps. L1 is the DNA ladder (Molecular sizes are indicated in kb). C (lane 7) is the control vector digested with the same enzyme.

### 3.3.4 Expression and Solubilisation of CHPV proteins as fusions with His and Strep tags

CHPV genes cloned as His (pLTA) and Strep (pCAK) tag fusions were transformed in BL21 cells for protein expression. The conditions for over-expression and solubilisation of these fusion proteins were standardized using different inducer concentrations and varying temperatures. Although, the recommended concentration of arabinose required for the expression of Strep fusion protein is 0.2% \cite{139}, an increase in the expression level and solubility was observed at a higher concentration of 0.5% for all viral proteins except G.
protein. Similarly, induction conditions for His fusion proteins were also standardized by expressing these proteins at 20 ng/ml, 50 ng/ml and 100 ng/ml of anhydrotetracycline. The optimum expression levels and increased solubility was observed for N, P and M protein at 20 ng/ml of anhydrotetracycline. G protein as Strep and His fusion remained insoluble at investigated inducer concentrations.

The induction conditions were further optimised by varying the induction temperature and duration. The proteins were expressed at 16 °C for 16 hours, 25 °C for 4 hours and 37 °C for 2 hours at optimized inducer concentrations (0.5% arabinose for Strep fusion and 20 ng/ml anhydrotetracycline for His fusion). The desired expression levels were observed at 25 °C and 37 °C for all four proteins. But, their levels remained undetectable at 16 °C. N, P and M proteins both as His and Strep fusions were found to be more soluble after induction at 25 °C [His-N; Figure 3.16 (b) lane 5, 3.17 (b) lane 2: His-P; Figure 3.16 (a) lane 4, 3.17 (a) lane 2: His-M; Figure 3.16 (d) lane 4, 3.17 (b) lane 4: Strep-N; Figure 3.18 (b) lane 3, 3.19 (b) lane 2: Strep-P; Figure 3.18 (a) lane 2, 3.19 (a) lane 1: Strep-M; Figure 3.18 (d) lane 4, 3.19 (b) lane 5]. G protein was insoluble i.e., remained in the cell pellet fraction [His-G; Figure 3.16 (c) lane 5, 3.17 (a) lane 4: Strep-G; Figure 3.18 (c) lane 5, 3.19 (a) lane 5] even with variations in temperature. The increased solubility at lower temperature can be accounted to the formation of lesser amount of protein aggregates at optimized expression levels [146]. All the proteins were observed at their expected sizes after analysis by SDS-PAGE and western blotting [His/Strep-N; 48 kDa, His/Strep-P; 34 kDa (observed at ~52 kDa due to aberrant mobility), His/Strep-M; 27 kDa, His/Strep-G; 70 kDa] [Figures 3.16 and 3.17 (His fusion proteins), 3.18 and 3.19 (Strep fusion proteins)].

G protein was observed to be insoluble irrespective of the tags, variations in inducer concentration and temperature conditions. Recently, Tao and co-workers described a protocol for the solubilisation of fusion proteins from inclusion bodies [140]. The protocol involved the usage of an anionic detergent, sarkosyl, as a solubilising agent at 10% concentration and addition of Triton X-100 and CHAPS for facilitating efficient purification by binding to glutathione resin. The protocol was adopted for the purification of G protein. However, the binding of G protein was not detected on the resin under these conditions. Hence, the protocol was modified and the cell pellet incubation with 10% sarkosyl overnight was followed by a dialysis step. Triton X-100 and CHAPS were not included after sarkosyl treatment. After all these modifications, G protein was observed to be soluble through this
method (Figure 3.20, lane 5). The solubilised fraction was dialysed to reduce the concentration of detergent to avoid hinderance with the purification of protein.

The sarkosyl treated and dialysed fraction of GST-G and lysates of GST-M were used for purification. These proteins were purified by affinity chromatography using glutathione sepharose beads and analysed by SDS-PAGE followed by Coomassie brilliant blue staining. Eluted fractions (E1 and E2) showed band at the size of 95 kDa for GST-G (Figure 3.21, lane 1 and 2) and at 52 kDa for GST-M protein (Figure 3.22, lane 2 and 3). Purification of N and P proteins has already been reported [20, 21]. The purification of CHPV G protein using a bacterial expression system has not been reported for any rhabdovirus. The solubilised proteins were used for investigating intraviral interactions among CHPV proteins as described in Chapter-5.

**Figure 3.16:** Solubilisation analysis of CHPV P, N, G and M proteins as His tag fusions

BL21 cells harbouring pLTA-N, pLTA-P, pLTA-M and pLTA-G plasmids were induced and cell pellets were collected before (BI) and after induction (AI). After induction cells were harvested, lysed and centrifuged. The soluble (supernatant, sup) and insoluble (pellet) fractions collected by centrifugation were analysed by SDS-PAGE. Panel (a), (b), (c) and (d) represent the solubilisation analysis of His tagged P, N, G and M proteins, respectively. Panel (a) lanes 2-5 represents, BI, AI, sup and pellet fractions for P protein solubilisation analysis, while Panel (b) lanes 2-5 indicate AI, BI, pellet and sup fractions for N protein. Panel (c) lanes 1, 2, 4 and 5 represents AI, BI, sup and pellet fractions for G protein solubilisation analysis, and Panel (d) lanes 2-5 indicate
AI, BI, sup and pellet fractions for N protein. Marker is the prestained Protein ladder (Fermentas; molecular sizes are indicated in kDa).

**Figure 3.17:** Solubilisation analysis of CHPV N, P, M and G proteins as His tag fusions by western blotting

Expressed and solubilised His-CHPV proteins were analysed by western blotting. The cell were harvested, lysed and centrifuged. The soluble (supernatant, Sup) and insoluble (pellet, Pl) fractions were analysed using anti-His monoclonal antibody. For panel (a) various samples are represented as; Pl and Sup from P in lane 1 and lane 2 respectively; Pl and Sup from G in lane 4 and lane 5 respectively. For panel (b) Sup and Pl from N in lane 2 and lane 3, respectively and Sup and Pl from M in lane 4 and lane 5, respectively. Marker represents the prestained protein ladder (Fermentas; molecular sizes are indicated in kDa).
Figure 3.18: Solubilisation analysis of CHPV P, N, G and M proteins as Strep tag fusions
BL21 cells harbouring pCAK-P, pCAK-N, pCAK-G and pCAK-M plasmids were induced and cell pellets were collected before (BI) and after induction (AI). After induction cells were harvested, lysed and centrifuged. The soluble (supernatant, Sup) and insoluble (Pellet) fractions collected by centrifugation were analysed by SDS-PAGE. Panel (a), (b), (c) and (d) represent the solubilisation analysis of Strep tagged P, N, G and M proteins, respectively. Panel (a) lanes 2-5 represents sup, pellet, AI and BI fractions for P protein solubilisation analysis, while Panel (b) lanes 2-5 indicate BI, AI, sup and pellet fractions for N protein. Panel (c) lanes 2-5 represents BI, AI, sup and pellet fractions for G protein solubilisation analysis, and Panel (d) lanes 2-5 indicate BI, AI, sup and pellet fractions for M protein. Marker is the prestained Protein ladder (Fermentas; molecular sizes are indicated in kDa).
Figure 3.19: Solubilisation analysis of CHPV N, P, M and G proteins as Strep tag fusions by western blotting
Expressed and solubilised Strep-CHPV proteins were analysed by western blotting. The cell pellets were
harvested, lysed and centrifuged. The soluble (supernatant, Sup) and insoluble (pellet, Pl) fractions were
analysed using anti-Strep monoclonal antibody. For panel (a) various samples are represented as; Sup and Pl
from P in lane 1 and lane 2 respectively; Sup and Pl from G in lane 4 and lane 5 respectively. For panel (b) Pl
and Sup from N in lane 1 and lane 2 respectively, and Pl and Sup from M in lane 4 and lane 5 respectively.
Marker represents the prestained protein ladder (Fermentas; molecular sizes are indicated in kDa).

Figure 3.20: Solubilisation of CHPV G protein as GST fusion using sarkosyl
BL21 cells expressing G protein as GST tag fusion were lysed and centrifuged. The pellet fraction was treated
with sarkosyl, dialysed and centrifuged. The supernatant and pellet fractions were analysed by SDS-PAGE.
Samples in SDS-PAGE are represented as; Lane 1: Pellet before Sarkosyl treatment; Lane 2: Sup before sarkosyl
treatment; Lane 4: Pellet after Sarkosyl treatment; Lane 5: Sup after Sarkosyl treatment;
**Figure 3.21:** Purification of CHPV Glycoprotein

*E. coli* BL21 cells expressing GST-G fusion protein were lysed and the soluble (supernatant) and insoluble (cell pellet) fractions were collected after centrifugation. GST-G protein was detected in the insoluble fraction and was solubilised using sarkosyl (lane 5). The solubilised fraction was dialysed (lane 4) and loaded on glutathione sepharose beads. Lane 1 and 2 represents eluate fractions E2 and E1 respectively. Flow through is represented in lane 3. Lane 6 is the protein ladder (molecular sizes are indicated in kDa).

**Figure 3.22:** Purification of CHPV Matrix protein

GST-M expressing cells were induced with IPTG and lysed. Cells were centrifuged and the soluble fraction (supernatant/cell lysate) was loaded onto the glutathione sepharose beads. Unbound fractions were removed by washing with 1X PBS called flowthrough (Lane 1)After elution of the bound protein, samples were analysed on 10% SDS-PAGE followed by Coomassie Brilliant Blue staining. Lane 2 and 3 indicate eluted fraction 1 (E1) and 2 (E2) respectively. Lane 4 is the protein ladder (molecular sizes are indicated in kDa).
3.4 Discussion

This work was aimed to generate the recombinant CHPV viral proteins, their solubilisation and purification for further interaction studies. The study provides the first instance on the effect of different tags, inducer concentrations, temperature and induction time on the expression and solubility of CHPV proteins. The expression conditions were optimized for all four (N, P, M and G) viral proteins with the aim to achieve maximum solubilisation.

CHPV M and G proteins were purified as GST fusions using bacterial expression system for the first time. The expression and solubilisation of G protein was particularly difficult in the present study. The purification of membrane proteins sometimes prove to be a difficult task because of their strongly hydrophobic nature which may form aggregates, leading to either rapid degradation or accumulation as inclusion bodies [147].

The CHPV G protein has previously been purified using baculovirus and Pichia expression systems [59, 60]. Baculovirus system is a complex and costlier system while Pichia, although a cheaper system requires standardization, has stability problems and is very time consuming. The use of bacterial expression system provides a simple, cheaper and rapid system for protein purification.

The fusion proteins generated in this study could be used as important materials for intraviral interaction analysis by pull down analysis, Co-IP assay and ELISA. The protein interactions among CHPV viral proteins was investigated using ELISA and GST pull down assays as described in Chapter-5.

These proteins can find applications for structural studies and their usage in the generation of antibodies and development of diagnostic assays. The protocols optimized can be used for the purification of membrane proteins of other viruses of Rhabdoviridae family as well.