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Cloning, Expression, And Purification Of Capsid Protein 3 Of Rice Tungro Spherical Virus And Its Preliminary Biophysical And Structural Characterization

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Abstract: Rice tungro disease (RTD) is a dangerous viral illness that has a significant impact on rice farming throughout Asia, resulting in significant crop yield losses. The infection is the result of a synergistic interaction between two viruses: Rice Tungro Bacilliform Virus (RTBV) and Rice Tungro Spherical Virus. The CP3 capsid protein is an important structural component of RTSV, playing a role in virion production, vector-mediated transmission, and host interactions. This study evaluates the translational efficiency and structural characteristics of the CP3 protein encoded by the RTSV cp3 gene. To achieve this, the gene was cloned into an SUMO-tagged expression system and heterologously expressed in Escherichia coli BL21(DE3). Using 1 mM IPTG at 18°C for 16 hours resulted in a 56-kDa soluble CP3 fusion protein. The recombinant protein was purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography, followed by size-exclusion chromatography [9]. Intrinsic fluorescence spectroscopy revealed a distinctive emission at 332 nm, indicating tryptophan residues in a hydrophobic interior. Transmission electron microscopy (TEM) revealed that pure CP3 protein self-assembled into VLPs with a consistent size of 30-35 nm and icosahedral symmetry. These findings show the protein's intrinsic ability to produce VLPs without the need for viral RNA, indicating its potential use in building diagnostic platforms and methods for analysing RTSV polyprotein function. Overall, the study increases our understanding of the RTSV capsid protein architecture and suggests opportunities for VLP-based biotechnological techniques.

Index Terms - Rice Tungro Disease (RTD); RTSV; RTBV; CP3 Capsid Protein; Protein Purification; Fluorescence Spectroscopy; Virus-like Particles (VLPs); Transmission Electron Microscopy; Plant Virology

I. INTRODUCTION

More than 50% of the world's population relies on rice (Oryza sativa L.) as a major dietary component.. However, its production is increasingly constrained by a variety of biotic stress factors, with viral infections being a major contributor to yield loss. Among these, Rice Tungro Disease (RTD) stands out as one of the most economically damaging viral diseases in South and Southeast Asia. RTD is caused by the co-infection of two different viruses: Rice Tungro Bacilliform Virus (RTBV) and Rice Tungro Spherical Virus. The latter, a member of the Sequiviridae family, is semi-persistently transmitted by the green leafhopper (Nephotettix virescens [2]), and it plays an important role in increasing disease severity and propagation.

RTSV encodes a number of structural and nonstructural proteins, with the CP3 capsid protein standing out for its various biological roles. CP3 is involved in the formation of the viral capsid, which allows for long-distance movement within the host and mediates interactions with plant and insect vector components. Understanding RTSV pathogenesis and transmission pathways requires a better understanding of CP3's structural and functional dynamics. Notably, capsid proteins such as CP3 frequently exhibit innate self-assembling characteristics, resulting in the creation of virus-like particles (VLPs) that physically resemble native virions but lack genetic material. These VLPs are gaining popularity as potential platforms for diagnostics, pesticide design, and nanobiotechnological applications.

Because it is difficult to extract adequate amounts of CP3 directly from infected plant material, heterologous production in microbial systems has emerged as the preferred technique. The insertion of SUMO (Small Ubiquitin-like Modifier) tags in Escherichia coli expression vectors has been demonstrated to improve the solubility and yield of recombinant proteins, especially those that tend to aggregate. Following production, these proteins can be effectively isolated using affinity chromatography and subjected to comprehensive structural investigation utilising techniques such as spectroscopy and electron microscopy.

In this paper, we describe the cloning, production, purification, and preliminary structural characterisation of the RTSV CP3 protein utilising an SUMO-fusion expression method in E. coli BL21(DE3). The isolated protein was analysed using Ni-NTA affinity chromatography, intrinsic fluorescence spectroscopy, and Transmission Electron Microscopy. The findings showed that CP3 efficiently produces monodisperse, icosahedral VLPs in vitro, validating its self-assembly capability and indicating its use in structural virology and next-generation plant virus-based diagnostics. These discoveries provide vital insights into CP3 architecture and set the framework for future biotechnological study.

II. MATERIALS AND METHODS

2.1 Molecular Investigation of the CP3 Capsid Protein

2.1.1 Cloning, overexpression and purification of cp3

The cp3 gene from Rice Tungro Spherical Virus (RTSV) was cloned into the SUMO-pRSFDuet-1 expression vector using the EcoRI and XhoI restriction sites. PCR amplification of the gene was performed in a 100 µL reaction mixture using 50 ng of viral genomic DNA, 0.5 mM of each primer, 0.2 mM dNTPs, and 5 units of Taq DNA polymerase. The thermal cycling technique included an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, and extension at 72°C for 60 seconds, culminating in a final extension step at 72°C for 5 minutes. The amplified products were purified using a standard PCR cleanup kit and incubated at 37 °C for 5 minutes.

Ligation was performed by combining the purified PCR product with the pre-digested SUMO-pRSFDuet-1 vector at a 3:1 insert-to-vector molar ratio in a 20 µL reaction mixture, followed by overnight incubation at 4 °C. For transformation, 2 µL of the ligation mixture was introduced into chemically competent E. coli BL21(DE3) cells via heat shock. Transformed colonies were selected on LB agar plates containing kanamycin and incubated at 37 °C overnight. Positive clones were identified through colony PCR and restriction enzyme digestion.

Initial expression analysis showed that CP3 was mainly accumulated in the form of inclusion bodies. To promote soluble expression, the gene was re-cloned into the SUMO-pRSFDuet-1 vector, which enhances solubility due to the presence of a SUMO (Small Ubiquitin-like Modifier) tag and an N-terminal hexahistidine (6×His) affinity tag. The final construct was sequence-verified by Eurofins Genomics (India) Pvt. Ltd., confirming 100% identity with the target cp3 sequence. The resulting expression cassette encoded a fusion protein consisting of an N-terminal 6×His tag, a SUMO domain, and the CP3 polypeptide. Two specific

proteolytic cleavage sites were engineered into the construct: one between the His tag and SUMO domain for thrombin cleavage, and another between the SUMO domain and CP3 sequence for SUMO protease-mediated release.

Optimization of Overexpression Conditions

Transformed Escherichia coli BL21(DE3) cells harboring the SUMO-pRSFDuet-1-cp3 construct were grown on LB agar plates containing 50 µg/mL kanamycin and incubated at 37 °C overnight. Confirmed recombinant colonies were selected to conduct small-scale expression trials aimed at optimizing conditions for enhanced CP3 protein production. Various induction setups were evaluated by modifying isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations (ranging from 0.05 mM to 1.0 mM), along with changes in incubation temperature and post-induction duration, using 10 mL culture volumes.

After induction, cells were harvested by centrifugation and washed with sterile saline. The resulting pellets were resuspended in 10 mL of lysis buffer composed of 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 0.05% (v/v) Triton X-100. To facilitate cell wall degradation, lysozyme was added to a final concentration of 100 µg/mL, and the suspension was incubated at room temperature for 30 minutes. Cell lysis was then carried out by ultrasonication using cycles of 3 seconds ON and 2 seconds OFF at 60% amplitude for a total duration of 1 hour, while maintaining the temperature at 4 °C to prevent thermal denaturation.

Following sonication, the lysate was centrifuged at 8,000 rpm for 30 minutes at 4 °C to separate the soluble protein fraction from the insoluble debris. The supernatant containing soluble proteins was collected, and expression levels under each tested condition were evaluated by resolving the samples on SDS-PAGE gels.

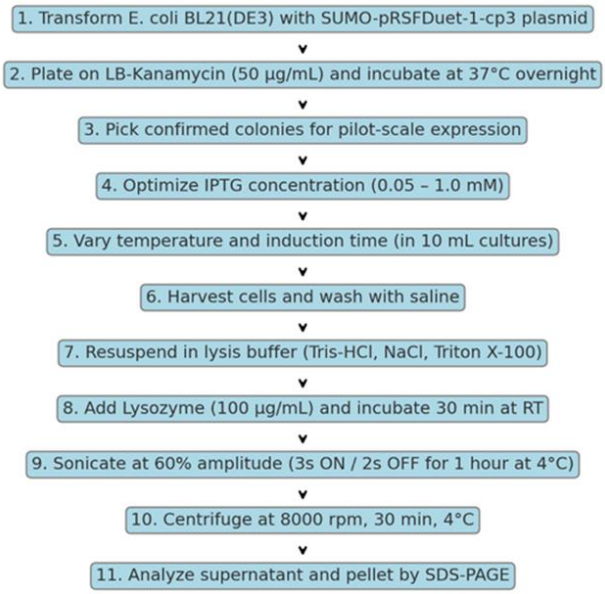

Figure 1: Flowchart of CP3 Expression Optimization Workflow

Purification of the cp3 protein

To enable large-scale expression of the CP3 protein, a 10 mL overnight culture of transformed E. coli BL21(DE3) was prepared and subsequently inoculated into 1 L of LB medium containing kanamycin (50 µg/mL). The culture was allowed to grow until it reached mid-logarithmic phase ($OD_{600} \approx 0.6$), at which point protein expression was induced by the addition of 1 mM IPTG. After induction, cells were harvested by centrifugation and resuspended in 60 mL of lysis buffer consisting of 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 10 mM imidazole, and 2% (v/v) Triton X-100. Lysozyme was added to the suspension to aid in cell wall breakdown, followed by mechanical disruption via sonication at 60% amplitude for 90 minutes, using a pulse pattern of 3 seconds ON and 2 seconds OFF.

The resulting cell lysate was clarified by centrifugation, and the supernatant—containing the soluble SUMO-tagged CP3 protein—was passed through a 0.22 µm filter to remove any residual debris. The filtered sample was then applied to a gravity flow column packed with 2.5 mL of Ni-NTA Sepharose resin. Non-specifically bound proteins were removed through sequential washing with two buffers: wash buffer I containing 7 mM imidazole and wash buffer II containing 10 mM imidazole, both formulated with 50 mM Tris–HCl (pH 8.0) and 500 mM NaCl.

The bound CP3 fusion protein was eluted in 1 mL fractions using an elution buffer composed of 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 500 mM imidazole. Protein concentration in the eluted fractions was quantified by measuring absorbance at 280 nm. The purity of the SUMO-CP3 fusion protein was assessed by 12% SDS-PAGE, followed by staining with Coomassie Brilliant Blue.

Size-Exclusion Chromatography and Tag Cleavage of cp3

Following affinity purification, the CP3-containing fractions were pooled and further purified utilising a fast protein liquid chromatography (FPLC) system and a size-exclusion chromatography (SEC) column. To prepare the column for sample injection, it was pre-conditioned with two litres of buffer containing 20 mM Tris-HCl (pH 8.0) and 100 mM NaCl. Protein was eluted at a flow rate of 1 mL/min, and fractions were recovered after two additional column volumes of buffer flow. The eluted samples were analyzed by SDS-PAGE to identify those containing the highest purity of the target CP3 protein.

The SEC fractions containing purified CP3 were pooled and subjected to enzymatic tag removal to separate the CP3 protein from its N-terminal His-SUMO fusion partner. Cleavage reactions were set up using His-tagged Ulp1 protease, targeting the specific site between the SUMO tag and the CP3 sequence. The reactions were prepared in 100 µL volumes with varying substrate-to-enzyme molar ratios of 25:1, 50:1, and 100:1 to determine the most efficient condition for complete digestion. The mixtures were incubated at 4 °C for 12 hours.

To separate the cleaved CP3 protein from the His-tagged SUMO fragment and Ulp1 protease, the reaction mixtures were passed through a second Ni-NTA affinity column. As the His-tagged components bound to the resin, the untagged CP3 was recovered in the flow-through using a buffer composed of 20 mM Tris–HCl (pH 8.0) and 100 mM NaCl. Bound fragments were subsequently eluted with a high-imidazole buffer (300 mM imidazole). The final CP3 protein, free of fusion tags, was verified for purity and size by SDS-PAGE.

2.1.2 Intrinsic Fluorescence Spectroscopy of CP3 Capsid Protein

Capsid proteins are known to contain a high proportion of aromatic amino acids, including tryptophan, tyrosine, and phenylalanine, which naturally exhibit fluorescence. Among these, tryptophan is particularly sensitive to changes in its local environment, making it a valuable intrinsic probe for monitoring protein conformation. Shifts in emission intensity, peak wavelength, or fluorescence quenching can provide insights into protein folding states, structural rearrangements, and the assembly or disassembly of capsid subunits. Therefore, intrinsic fluorescence serves as a powerful technique for tracking capsid maturation processes. In addition, alterations in the polarity or hydrophobicity of the surrounding environment during protein assembly can influence the fluorescence signature, enabling assessment of conformational states through both intrinsic signals and external fluorescent probes.

Instrumentation and Data Acquisition

Fluorescence spectra were recorded using an excitation wavelength of 280 nm, which excites both tryptophan and tyrosine residues. Emission was scanned across a range of 300–400 nm, with a slit width of 5 nm and a scan speed of 120 nm/min. The photomultiplier tube voltage was set at 700 V to ensure optimal sensitivity. Prior to measuring the protein sample, a baseline scan was obtained using phosphate buffer under identical settings. The buffer spectrum was subtracted from the raw protein data to produce the corrected emission profile.

The native CP3 protein showed a distinct emission maximum between 330 and 340 nm, consistent with tryptophan residues being buried within a hydrophobic core—a hallmark of properly folded proteins. A summary of the peak emission characteristics is provided in Table 1, and the corresponding emission spectra are illustrated in Figure 6. All measurements were performed using the FL Solutions 4.0 software on a Hitachi F-7000 fluorescence spectrophotometer.

2.1.3 Transmission Electron Microscopy (TEM) imaging

For TEM sample preparation, 5 µL of purified CP3 protein was dispensed onto a glow-discharged carbon-coated grid (discharged at 15 mA for 20 seconds) and allowed to adsorb for one minute on Parafilm. Excess sample was carefully removed using Whatman No. 1 filter paper. Negative staining was carried out by applying 5 µL of a 2% (w/v) uranyl acetate solution onto the grid for 30 seconds, after which the excess stain was gently wicked off using filter paper. The grid was then left to air dry for 30 minutes before imaging.

TEM Data Acquisition

Electron microscopy was conducted using a Talos L120C transmission electron microscope (Thermo Fisher Scientific), operated at an accelerating voltage of 120 keV. Image capture was performed with a CETA-M CMOS camera (4K × 4K resolution). The stained sample grids were inserted into the instrument, and high-resolution images were obtained under varying magnification settings using the Velox imaging software (Thermo Fisher Scientific).

III. RESULTS

3.1 Molecular Investigation of the CP3 Capsid Protein

3.1.1 Cloning of the *cp3* Gene into the Expression Vector

The *cp3* gene was successfully amplified from the SUMO-pRSF-Duet1–*cp3* plasmid using gene-specific primers tailored for subcloning. The resulting PCR product was approximately 1.46 kb in size, as confirmed in Figure 2. The amplified fragment was digested with the appropriate restriction enzymes and ligated into the SUMO-pRSF-Duet1 vector. This ligation resulted in the formation of a recombinant plasmid, termed SUMO-*cp3*, with an overall size of approximately 4.8 kb.

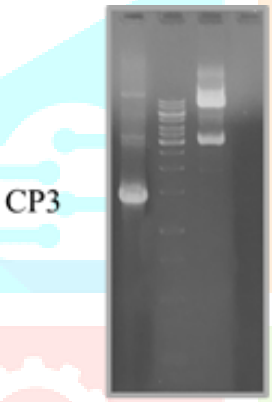

Figure 2: PCR amplification of RTSV cp3 (1.46kb)

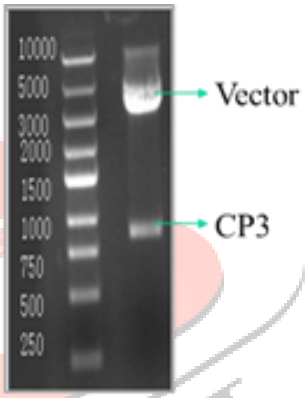

Figure 3: Restriction double digestion of RTSV cp3 (894bp) in SUMO-pRSFDuet-1

Optimization of cp3 expression conditions

A series of experimental trials were performed to determine the most effective conditions for maximizing both the expression and solubility of the *cp3* protein. Various IPTG concentrations were tested, with 1 mM found to produce the highest expression yield. Additional refinements to the induction protocol revealed that incubating the culture at 18 °C for 16 hours post-induction yielded optimal results for soluble protein production.

Based on these findings, the SUMO-*cp3* construct was expressed under the optimized condition of 1 mM IPTG induction at 18 °C for 16 hours. To evaluate protein expression, equivalent volumes of lysate supernatants from both induced and non-induced cultures were analyzed on a 12% SDS-PAGE gel. A distinct protein band observed in the induced sample confirmed the efficient and soluble expression of *cp3* under the established conditions.

Purification of cp3 by affinity and size-exclusion chromatography

The initial purification of the CP3 protein was performed using nickel-NTA affinity chromatography, which specifically binds the His-tag present in the SUMO-tagged fusion protein. Analysis of the eluted fractions via SDS-PAGE revealed a strong band at approximately 56 kDa, consistent with the predicted molecular weight of the SUMO-CP3 construct (see Figure 4). The inclusion of the hexahistidine-SUMO tag greatly enhanced both solubility and purification efficiency.

To further enhance the purity and remove residual salts and potential aggregates, the affinity-purified protein was subjected to size-exclusion chromatography (SEC). The SEC chromatogram displayed a single, sharp elution peak, suggesting that the CP3 protein was monomeric and structurally uniform (Figures 4 and 5). Subsequent SDS-PAGE analysis of these peak fractions confirmed the presence of a clean, distinct 56 kDa band, verifying both the purity and structural integrity of the CP3 protein.

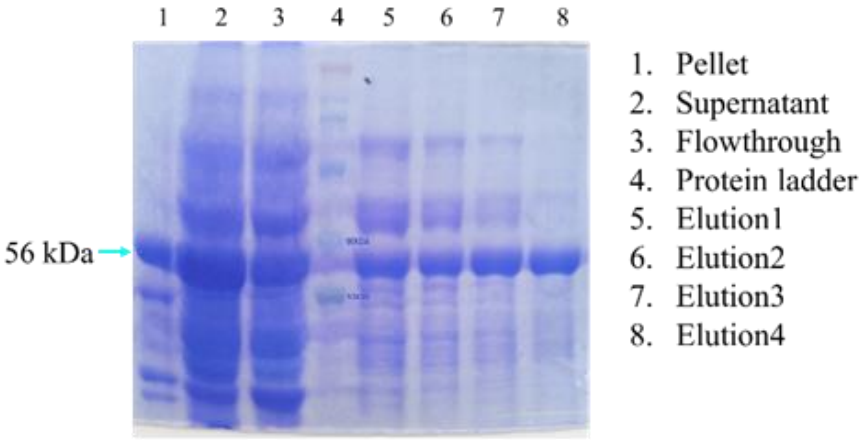

Figure 4: SDS-PAGE confirmation of cp3 elutions after SEC

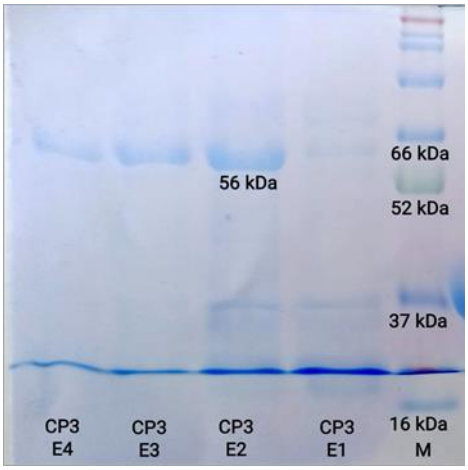

Figure 5: SDS–PAGE image of

3.1.2 Fluorescence Spectroscopy Studies

A distinct emission maximum within the 330–340 nm range was observed, which is indicative of tryptophan residues located in hydrophobic regions—characteristic of proteins in their native, folded conformation. The fluorescence peak values are compiled in Table 1 and graphically depicted in Figure 6. All spectral data were acquired using the FL Solutions 4.0 software on a Hitachi F-7000 fluorescence spectrophotometer.

Table 1: Fluorescence characteristics of capsid proteins

Condition	Expected peak λ (nm) (Approx.)	Resulted λ max (nm)	Fluorescence Intensity	Interpretation
Native (folded)	330–335	332	High	Tryptophan in hydrophobic core
Partially unfolded	340	340	Reduced	Tryptophan partially exposed
Fully denatured	350–355	355	Low or quenched	Tryptophan in polar environment (solvent)

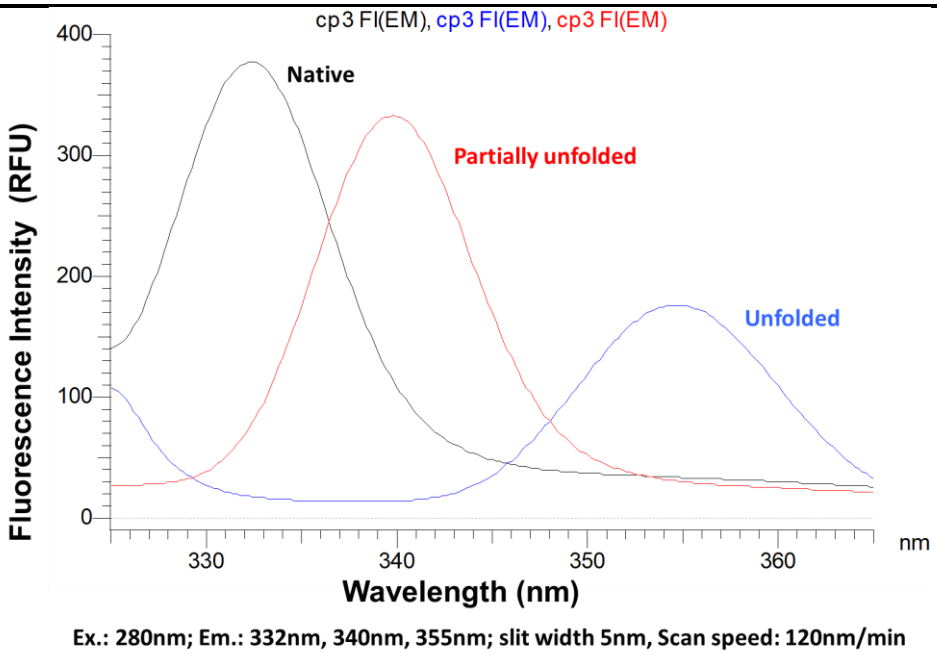

Figure 6: Intrinsic Fluorescence Spectroscopy of a Capsid Protein

3.1.3 Transmission Electron Microscopy (TEM) Imaging

TEM-Based Analysis of CP3 Capsid Protein Assembly

Transmission Electron Microscopy (TEM) was employed to evaluate the in vitro self-assembly characteristics of the CP3 capsid protein derived from *Rice Tungro Spherical Virus* (RTSV). As shown in Figure 7, TEM imaging revealed uniformly distributed, spherical particles measuring approximately 30 to 35 nm in diameter—dimensions consistent with previously reported sizes of RTSV virions. These particles exhibited circular symmetry and a dense outer boundary, features indicative of successful capsid-like oligomerization.

The consistent size and morphology observed across the field suggest that CP3 subunits self-assembled efficiently and homogeneously. Notably, the particles lacked internal nucleic acid content, implying that CP3 assembly occurred independently of viral RNA—a hallmark of virus-like particle (VLP) formation in recombinant systems expressing plant virus capsid proteins. The symmetrical architecture closely resembled icosahedral geometry [11], reinforcing their classification as VLPs and mirroring the structural profile of authentic RTSV capsids.

The micrograph's 100-nm scale bar confirms the size range of the formed protein complexes. These findings emphasise the structural significance of CP3 in viral particle assembly, as well as its potential application in the development of VLP-based systems for diagnostics, immunological tests, and pesticide administration. Future studies could include high-resolution three-dimensional structural investigation with cryo-electron microscopy and molecular dynamics simulations to refine epitope mapping and validate computational models.

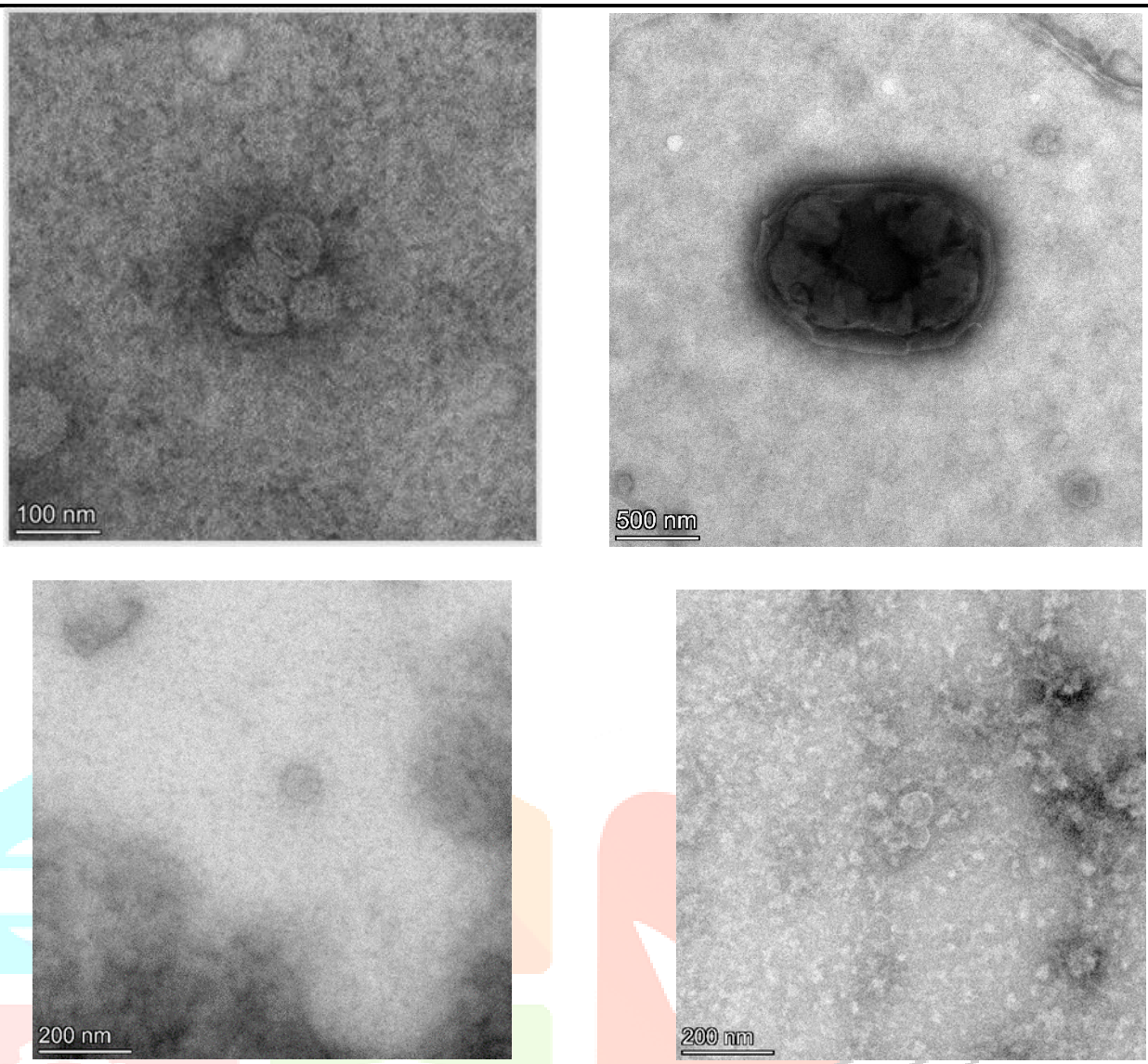

Figure 7: Cryo-TEM images showing uniform, spherical CP3 particles exhibiting icosahedral symmetry.

IV. DISCUSSION

This study effectively expressed and structurally evaluated the CP3 capsid protein from Rice Tungro Spherical Virus (RTSV), providing important insights into its self-assembly behaviour and possible biotechnological uses. The cp3 gene was cloned into an SUMO-fusion expression vector and inserted into Escherichia coli BL21(DE3). Optimal induction conditions (1 mM IPTG at 18 °C for 16 hours) resulted in a 56 kDa soluble fusion protein with increased yield and stability. [7,8].

The produced protein was purified using Ni-NTA affinity chromatography, followed by size-exclusion chromatography (SEC), resulting in a highly pure and monodispersed CP3 protein population [9]. The presence of a single, sharp SEC peak indicated the monomeric structure and correct folding. Intrinsic fluorescence spectroscopy confirmed the presence of tryptophan residues in a hydrophobic core, indicating a well-folded native shape. The emission maxima at 332 nm supports this result.

Transmission Electron Microscopy (TEM) showed that pure CP3 protein self-assembled into spherical virus-like particles (VLPs) of 30-35 nm in diameter. These structures exhibited icosahedral symmetry [11], which closely resembled the morphology of native RTSV virions. The absence of encapsidated nucleic acids proved that the particles were genuine VLPs generated independently of viral RNA, a frequent characteristic found in recombinant capsid protein expression systems.

CP3's ability to create stable, symmetrical VLPs in vitro demonstrates its potential as a framework for the development of virus-based nanomaterials such as diagnostic probes and antigen delivery platforms. Furthermore, using an SUMO fusion tag improved solubility and prevented protein aggregation, which is a common difficulty when expressing plant viral proteins in E. coli[7,8].

Looking ahead, enhanced structural studies combining cryo-electron microscopy and computer modelling [13] could provide high-resolution insight into the capsid's architecture, allowing for the discovery of surface-exposed epitopes for immunogenic or functional modification. Future research should also look into the

dynamics of CP3 interactions with host and vector proteins, which will help us better understand its involvement in viral transmission and pathogenesis. Overall, this study provides a solid platform for CP3-based biotechnological advancements and adds to our understanding of plant virus assembly mechanisms.

V. CONCLUSION

This paper effectively describes the molecular cloning, production, purification, and structural characterisation of the CP3 capsid protein from the Rice Tungro Spherical Virus (RTSV) utilising an SUMO-tagged expression platform in Escherichia coli. To produce a soluble 56 kDa fusion protein, expression was optimised and purified to near homogeneity by affinity and size-exclusion chromatography. Intrinsic fluorescence spectroscopy indicated that CP3 maintained a folded, native-like shape [10], while Transmission Electron Microscopy (TEM) demonstrated its ability to self-assemble into spherical, icosahedral virus-like particles (VLPs).

The findings clearly illustrate CP3's innate ability to construct higher-order assemblies in the absence of viral RNA, indicating its potential for usage in VLP-based technologies such as diagnostic assays, herbicide production, and nanoscale delivery systems. The SUMO fusion approach significantly increased the solubility [7,8] and expression yield of this structurally important plant virus protein. Future research could focus on comprehensive structural elucidation, interaction studies with host and vector components, and the use of CP3 VLPs in translational and synthetic virology.

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