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Molecular modeling and conformational analysis of native and refolded viral genome-linked protein of Cardamom mosaic virus

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The viral genome-linked protein (VPg) of Potyviruses is covalently attached to the 5' end of the genomic RNA. Towards biophysical characterization, the VPg coding region of Cardamom mosaic virus (CdMV) was amplified from the cDNA and expressed in *E. coli*. Most of the expressed VPg aggregated as inclusion bodies that were solubilized with urea and refolded with L-arginine hydrochloride. The various forms of CdMV VPg (native, denatured and refolded) were purified and the conformational variations between these forms were observed with fluorescence spectroscopy. Native and refolded CdMV VPg showed unordered secondary structure in the circular dichroism (CD) spectrum. The model of CdMV VPg was built based on the crystal structure of phosphotriesterase (from *Pseudomonas diminuta*), which had the maximum sequence homology with VPg to identify the arrangement of conserved amino acids in the protein to study the functional diversity of VPg. This is the first report on the VPg of CdMV, which is classified as a new member of the Macluravirus genus of the Potyviridae family.

Keywords: Viral genome-linked protein, Cardamom mosaic virus, Potyvirus, Inclusion bodies

Cardamom mosaic virus (CdMV) is a member of genus Macluravirus of family Potyviridae[1]. The members of Potyviridae family have flexible rod-shaped particles packing a single-stranded, polyadenylated, positive-sense genomic RNA[2]. The 5'end of viral RNA has VPg (viral protein genome-linked), covalently attached through a Tyr residue[3-5].

Among positive-sense RNA viruses, families like Luteoviruses, Picornaviruses, Comoviruses, Nepoviruses and Potyviruses are known to contain 5'-terminal VPg. The N-terminal VPg domain is slowly released from nuclear inclusion protein a (NIa) by the proteolytic activity of the C-terminal proteinase domain (NIa-Pro) in the course of potyvirus infection[6]. Potyviral VPg is a small (22-24 kDa) multifunctional protein. VPg interacts with other proteins of the virus and the host[7-9]. It contains a nucleotide triphosphate (NTP)-binding site and binds RNA in a sequence non-specific manner[10]. VPg participates in virus replication[11], translation of viral genome[7,12], virus cell-to-cell[13] and vascular movement[14-17].

The structure of plant virus VPg has been difficult to obtain due to the intrinsic structural disorder of these proteins. These proteins lack a rigid structure under physiological conditions and in their native fold, but instead have an ensemble of folds rapidly changing from one to another. The structural flexibility has been proposed to enable VPg to carry out a variety of functions. Potato virus Y (PVY) of Potyviridae has recently been reported to be a highly disordered protein[18] and Potato virus A (PVA) has a molten globule-like loose tertiary structure with a hydrophobic domain[19]. Although the three-dimensional (3D) structure of the VPg of plant and animal viruses has not been determined so far, the 3D model of PVY VPg has been built based on the structure of malate dehydrogenase[20].

In this study, we report the homology modeling of CdMV VPg based on the structure of phosphotriesterase of *Pseudomonas diminuta* and the cloning, expression and refolding of CdMV VPg from *E. coli*. Various forms of VPg (refolded and native) have been purified by Ni-NTA affinity chromatography. The conformational analysis of refolded and native VPg has been studied by using fluorescence spectroscopy and circular dichroism.

Materials and Methods

Cloning of VPg coding region

VPg coding region of CdMV (0.5 kb) was amplified from pJS5 (which contained a 2.9 kb

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fragment comprising partial cytoplasmic inclusion protein (CI), complete 6K2, VPg and nuclear inclusion protein a (NIa) protease coding region of CdMV (Accession no. for VPg: AJ550378) with VPg.F (5'-*GGATTCC*ATGAAAGGGAAGCATTTG-3') and VPg.R (5'-*AAGCTT*TTACTCCAGAGTTTG CTGTGG-3') primers (purchased from Sigma-Aldrich, India) by using Taq DNA polymerase (New England Biolab, USA). The amplicon was cloned in pXcmkn12 vector[21] (pXcm-VPg). The insert was released from pXcm-VPg with *Bam*HI (included in VPg.F primer, marked in itatics) and *Hind*III (included in VPg.R, marked in itatics) and cloned at the corresponding sites of pHT7[22] (pVPg).

Expression of VPg

pVPg was transformed into BL21 (DE3) *E. coli* cells. For expression, 1% of an overnight grown culture of BL21 cells harboring pVPg was inoculated in LB medium and the culture was grown till 0.6 optical density at 600 nm ($O.D_{600}$) at 37°C. The protein expression was induced by the addition of IPTG (isopropyl-β-D-1-thiogalactopyranoside) to a final concentration of 0.2 m*M* and the cells were further grown for 2.5 h at 37°C.

Purification of expressed VPg in native form

The VPg expressing cell pellets were washed with water, resuspended with native buffer (10 mM Tris, pH 7.5, 1 m*M* EDTA and 1 *M* NaCl) and sonicated (SONICS, Vibra cell 300 W) giving 10 pulses for 10 s each and then incubated for 30 min. The cell extract was spun at 10,000 *g* for 10 min and the supernatant was mixed with Ni-NTA (nickel-nitrilotriacetic acid) agarose and incubated for 30 min. The column was washed with increasing concentrations of imidazole (10-100 m*M*) and finally the bound protein was eluted with 200 m*M* imidazole.

Solubilization of inclusion bodies, refolding and purification of refolded VPg

The pellet (inclusion bodies) from native purification was washed with 0.8% (w/v) saline and washed twice with TTN buffer (50 m*M* Tris, pH 8.5, 0.1 *M* NaCl and 2% Triton X-100) and TN buffer (50 m*M* Tris, pH 8.5 and 0.1 *M* NaCl). After each wash, the sample was centrifuged at 7000 rpm for 7 min at 20°C. Inclusion bodies were solubilized in Tris-urea buffer (50 m*M* Tris, pH 8.5, 0.1 *M* NaCl and 8 *M* urea). Solubilization was carried out for 5 h on moderate shaking at 37°C and the suspension was centrifuged at 13,000 rpm for 20 min at 20°C to obtain unfolded VPg in the supernatant. Refolding buffer (50 m*M* Tris, pH 8.5, 0.1 *M* NaCl, 10% (v/v) glycerol, and 0.5 *M* L-arginine hydrochloride) was pre-chilled and kept at 4°C with stirring, into which the unfolded VPg was slowly added in 1:10 protein to buffer ratio. The solution was incubated at 4°C for 36 h. The protein solution was subjected to centrifugation at 12,000 rpm for 30 min at 4°C to remove small aggregates and particulate matter. The supernatant of refolded protein was purified by passing through a Ni-NTA agarose column. The column was washed with increasing concentrations of imidazole (10-25 m*M*) and finally the bound protein was eluted with 50 m*M* imidazole.

Ni-NTA purification of denatured VPg

pVPg expressing cell pellet was sonicated with sonication buffer (SB) (8 *M* urea in 0.1 *M* Tris, 0.01 *M* NaH_2PO_4, pH 8.0) and incubated for 30 min. The cell extract was passed through a Ni-NTA column and the column was washed with increasing concentrations of imidazole (10-100 m*M*) and finally the bound protein was eluted with 200 m*M* imidazole.

Production of anti-VPg antiserum

A sensitizing dose of 0.7 mg of purified VPg was given intramuscularly to a rabbit with complete adjuvant, followed by two subcutaneous injections with 500 µg of protein in incomplete adjuvant. A final dose of 500 µg of VPg was given intra-dermally with incomplete adjuvant. All injections were given at weekly intervals. Serum was collected ten days after the final injection.

Western blotting

The purified VPg and lysate of BL21 cells harboring pHT7 were subjected to Western analysis with a polyclonal antiserum raised against VPg as the primary antiserum.

Concentrating VPg

The purified denatured, refolded and native forms of VPg were pooled and concentrated by using an ultrafiltration concentrator with 10 kDa molecular weight cut-off membrane (Amicon). The filtrate was spun at 12,000 rpm for 10 min and supernatant was used for fluorescence spectroscopic and circular dichroism (CD) studies.

Fluorescence spectroscopy

120 µg of VPg (native, denatured and refolded) was used for measuring the O.D in the F-2500 FL

Spectrophotometer (Hitachi). Emission spectra from 250 to 800 nm were scanned after an excitation at 280 nm.

Circular dichroism (CD) analysis

The CD analysis was recorded by using JASCO spectropolarimeter J-810 (163-900 nm). Wavelength scan was done from 250 to 200 nm with protein concentration of 50 µg/ml. A cuvette with 0.1 cm path length was used with the parameters: resolution 0.5 nm, bandwidth 1.0 nm, sensitivity 50 mdeg, response 4 s and accumulation 3.

Modeling

The secondary structure of VPg (178 residues) was predicted by using Jpred and PHD programs. The structure of phosphotriesterase (1PSC_B) of *Pseudomonas diminuta* was chosen as the template. Initially the structure was modeled using homology module of Biosuite[23]. The modeled protein from Biosuite package was loaded on to Insight II and homology based model was built by Biosym module of Insight II (Accelerys, Inc). Energy minimization was done using Discover module of Insight II.

Results and Discussion

The VPg coding region of CdMV from viral cDNA was amplified and cloned in pHT7, an *E. coli* expression vector. VPg was found as both soluble and insoluble forms at 37°C with 0.2 m*M* IPTG concentration. The soluble form of VPg was taken in Tris buffer containing 0.1 *M* NaCl and 1 m*M* EDTA and purified by passing through a Ni-NTA column (Fig. 1). The insoluble form (inclusion bodies) was denatured with 8 *M* urea, which upon Ni-NTA purification gave a pure protein in 200 m*M* imidazole fraction (data not shown), which was used to raise a polyclonal antiserum. VPg protein expressed in BL21 cells gave a positive reaction with the above antiserum (Fig. 2).

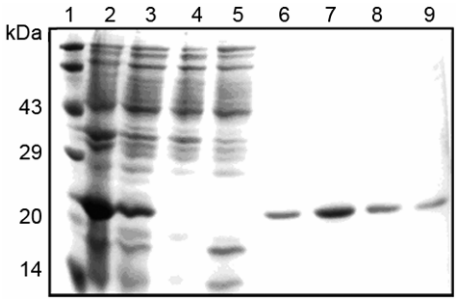

Fig. 1—Purification of native VPg [Lane 1, protein marker; lane 2, flowthrough; lane 3, pellet; lane 4, supernatant; elution with 25 m*M* (lane 5), 50 m*M* (lane 6), 75 m*M* (lane 7), 100 m*M* (lane 8) and 200 m*M* (lane 9) imidazole]

A number of combinations of solubilizing agents (urea, guanidium hydrochloride sodium dodecyl sulphate, TritonX-100), refolding buffers (different concentrations of arginine hydrochloride, glycerol and NaCl) and methods of refolding (in column and solution) were combined to optimize the conditions for refolding. The buffer containing 10% glycerol and 0.5 M arginine hydrochloride was found to be suitable to refold the denatured VPg. Arginine has been used to refold a few recombinant proteins produced in *E. coli* as inclusion bodies[24,25]. The effect of arginine on protein refolding is considered to be its ability to suppress aggregation of folding intermediates. Glycerol probably helps in preventing the competing process of aggregation during refolding, possibly by enhancing protein stability[26].

The native and refolded VPg differed in their efficiency to bind to Ni-NTA. In the case of native (Fig. 1) and denatured VPg (data not shown), the pure protein started eluting in a range (50-200 mM) of imidazole concentrations, whereas the refolded VPg was completely eluted even with 50 mM imidazole (data not shown). This could be due to the difference in the exposure of N-terminal His tag in VPg, which ultimately affects the binding to the Ni-NTA column.

In order to study the conformational differences between various forms of VPg (native, denatured and refolded), the proteins were diluted in respective buffers and O.D. was measured (Fig. 3). The denatured protein gave a peak at 368 nm and when this protein was refolded, the peak shifted to 360 nm (with glycerol) and 354 nm (without glycerol). To compare with the refolded form, spectrum of the native form of the protein was taken, which gave a peak at 380 nm (Fig. 3). These conformational variations could have influenced the difference in exposure of His tag in a VPg molecule, which affects

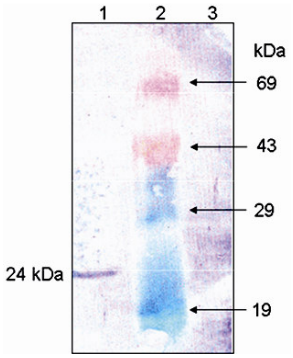

Fig. 2—Western blot of VPg [Lane 1, purified VPg; lane 2, prestained protein marker and lane 3, lysate of BL21 cells having pHT7]

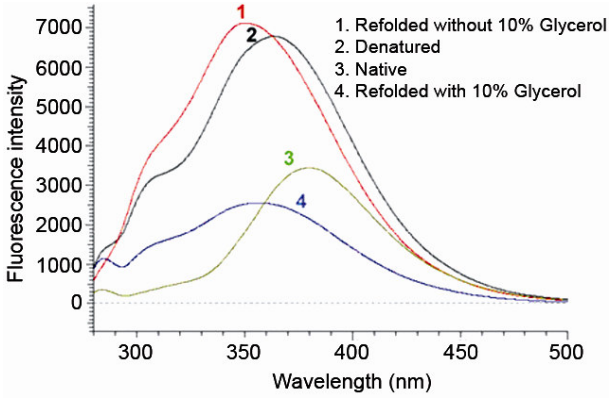

Fig. 3—Fluorescent emission spectra for an excitation at 280 nm [Emission was scanned between 250 and 800 nm. Native, denatured, refolded VPg with and without 10% glycerol gave peaks at 380, 368, 360 and 354 nm, respectively]

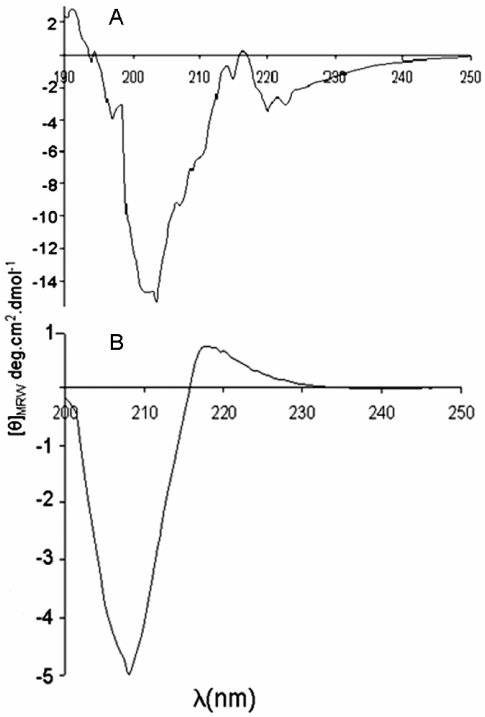

Fig. 4—CD spectrum of native (A) and refolded VPg (B)

the binding of His tag to the Ni-NTA column and so different forms of VPg eluted in different concentration of imidazoles.

The CD study was also performed to know the secondary structure of various forms of VPg. The native (Fig. 4A) and refolded (Fig. 4B) VPg showed a CD spectrum with a strong negative peak near 204 nm and 208 nm, respectively. The negative peak obtained for VPg in CD spectrum was typical of unordered or disordered proteins, which had little, ordered structures[27]. Earlier, the disordered nature of VPg has

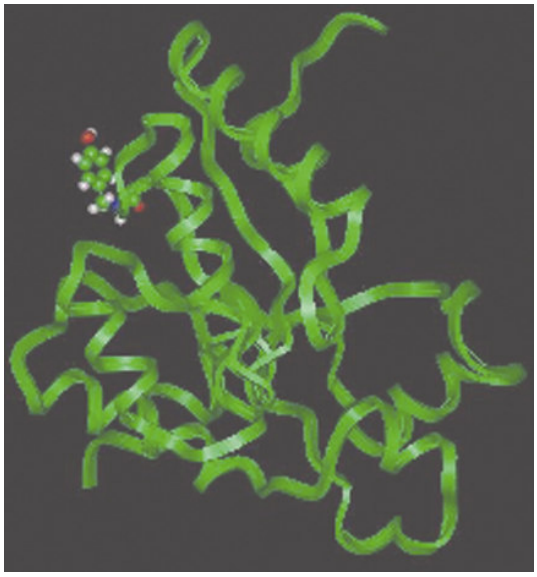

Fig. 5—Hypothetical model of CdMV VPg [The green traces the polypeptide backbone. A CPK (Corey-Pauling- Koltun) space-filling representation is used to show the Tyr-64 position]

been reported for Sesbania mosaic virus VPg[28], PVY VPg[18] and PVA VPg[19]. Similar to CdMV VPg, the far-UV spectrum of PVA VPg has also shown some typical features for intrinsically disordered proteins, namely a minimum at 203 nm and a negative shoulder near 222 nm in CD spectroscopic analysis[19]. The presence of a hydrophobic core domain and a loose tertiary structure among the VPg protein could cause the partially disordered nature of VPg[19].

The phosphotriesterase (1PSC_B) of *P. diminuta* was identified as the best structural template for the molecular modeling of CdMV VPg[29]. Although the N- and C-terminus of protein were modeled based on the template structure, there was no serious steric hindrance in the structure. It was also confirmed that secondary structure of these two termini was quite similar based on Jpred and PHD secondary structure prediction programs.

The molecular model of CdMV VPg is shown in Fig. 5. A total of eight helices, five ß-strands and five turns were present in the structure. The Tyr residue at position 64 of VPg, which is conserved among other Potyviruses was found in a coil region connecting a helix and ß-strand near the N-terminus (Fig. 5). A Lys residue at position 40 was buried within the structure, which was unusual because charged residues are preferably present on the surface of the structure[19]. Further refinement of the structure did not give any major steric hindrances. Therefore, it could be predicted that Lys residue was present in the major active site of the protein.

The model of CdMV VPg was compared with the VPg of PVY which was also modeled using the Insight II program of Biosym package with malate dehydrogenase as the template[18]. This model also contained several helical fragments and some of the solvent inaccessible residues were located on the buried sites of these helices (data not shown). The Tyr 64 of CdMV VPg, which is also conserved in PVY VPg at 63rd amino acid position was located at the N-terminus of the buried helix with only the hydroxyl group exposed.

Conclusion

Most of the VPg of CdMV was expressed as aggregated proteins in *E. coli*. It was solubilized and refolded. The various forms of CdMV VPg (native, denatured and refolded) were purified and the conformational variations between these forms were observed with fluorescence spectroscopy and CD. Native and refolded CdMV VPg showed unordered secondary structure in the CD spectrum. The model of CdMV VPg was built based on the crystal structure of phosphotriesterase from *Pseudomonas diminuta*. A total of eight helices, five ß-strand and five turns were present in the structure. The Tyr residue at position 64 of CdMV VPg which is conserved among other potyviruses was present in a coil region, suggesting the structural conservation of the Tyr that is crucial for the function of potyviral VPg proteins

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