Purification and biochemical characterization of a monomeric form of papaya mosaic potexvirus coat protein

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Abstract

Papaya mosaic virus (PapMV) is a flexuous rod shape virus made of 1400 subunits that assemble around a plus sense genomic RNA. The structure determination of PapMV and of flexuous viruses in general is a major challenge for both NMR and X-ray crystallography. In this report, we present the characterization of a truncated version of the PapMV coat protein (CP) that is suitable for NMR study. The deletion of the N-terminal 26 amino acids of the PapMV CP (CP_{27–215}) generates a monomer that can be expressed to high level and easily purified for production of an adequate NMR sample. The RNA gel shift assay showed that CP_{27–215} lost its ability to bind RNA in vitro, suggesting that the multimerization of the subunit is important for this function. The fusion of a 6\textsuperscript{H}E tag at the C-terminus improved the solubility of the monomer and allowed its concentration to 0.2 mM. The CD spectra of the truncated and the wild-type proteins were similar, suggesting that both proteins are well ordered and have a similar secondary structure. CP_{27–215} was \textsuperscript{15}N labeled for NMR studies and a 2D \textsuperscript{1H}–\textsuperscript{15}N-HSQC spectrum confirmed the presence of a well-ordered structure and the monomeric form of the protein. These results show that CP_{27–215} is amenable to a complete and exhaustive NMR study that should lead to the first three-dimensional structure determination of a flexuous rod shape virus.

Keywords: Papaya mosaic virus; Coat protein; Monomer; Potexviruses; NMR

Papaya mosaic virus (PapMV) is a member of the large family of the Flexiviridea\textsuperscript{1} and the gender potexvirus. The virus harbors a flexuous rod shape of 500 nm in length and 14 nm in diameter. There are 1400 subunits of the viral coat protein (CP) in each rod assembled around a plus strand genomic RNA of 6656 nucleotides \textsuperscript{2}. PapMV CP is a protein of 215 amino acids having a molecular weight of 23 kDa. Many studies about the in vitro reconstitution of PapMV nucleocapsid-like particles (NLPs) were done using CP extracted from purified virus treated with the acetic acid degradation method \textsuperscript{2–7}. We have recently shown that PapMV CP can be expressed to high level in \textit{Escherichia coli} and form NLPs that are similar to the WT virus \textsuperscript{8}. Twenty percent of the protein was found as NLPs while the remaining was found as a multimer of 450 kDa (20 subunits) that forms a disk \textsuperscript{8}. The disks were shown to be the basic building block of the NLPs in vitro. The assembly process occurs probably by a similar mechanism in planta.

Alignment of the amino acid sequences of filamentous viruses showed two distinct groups: the flexuous group made of the potex-, carla-, clostero-, poty- and bymoviruses and

\textsuperscript{1} Abbreviations used: PapMV, papaya mosaic virus; CP, coat protein; NLPs, nucleocapsid-like particles; PVX, potexvirus X; NMV, narcissus mosaic potexvirus; TMV, tobacco mosaic virus; EM, electron microscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; EMSA, electrophoresis mobility shift assay; PVBV, pepper vein banding virus.

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the group of rigid rod made of the tobamo-, tobra-, hordei-
, and furoviruses [9]. A comparison between the Raman
spectra of two flexuous rods (potato potexivirus X (PVX)
and narcissus mosaic potexivirus (NMV)) and a rigid rod
(tobacco mosaic virus (TMV; tobamovirus)) revealed that
proteins of both types are rich in α-helices but that potex-
viruses have longer loops between the helices [10]. Potexvi-
ruses coat proteins are also more hydrated which probably
contribute to their flexibility [10]. A similar study on PVX
also revealed that the N- and C-terminal ends of the coat
protein are located close to the surface of the virus [11,12].
An X-ray diffraction study of PVX showed 8.75 CP per
turn of helix of the virus particle on which 4–5 nucleotides
are associated [13]. Finally, the crystallization and prelimi-
ary X-ray analysis of PapMV did not lead to the resolu-
tion of the structure of the virus due to the heterogeneity of
the sample used for crystal growth and the small size of the
crystals [14].

PapMV CP exists in E. coli as disks and NLPs, two mul-
timeric states that are not amenable to NMR structure
determination. The three-dimensional structure determina-
tion approach by NMR typically requires a total molecular
weight of less than 60 kDa and concentrated samples. It is
therefore necessary to produce an uniform and low molecu-
lar weight sample. In this report, we present a monomeric
form of the PapMV CP, CP27–215 that has a similar second-
ary structure than the wild-type protein. Preliminary bio-
chemical and NMR data confirmed that the protein is
amenable under this form to an extensive 3D NMR struc-
tural study.

Materials and methods

Cloning and expression of the recombinant proteins

The PapMV CP gene CP6–215 was described elsewhere
[8]. The truncated version of PapMV CP, CP27–215, CP46–215,
and CP60–215 were amplified by PCR from the clone CP6-215.
The following forward primers were used: CP27–215, 5’-agt
cccatggaatccgccatacctgtcag-3’, CP46–215 5’-agctccatgtgct-
getaaggctcagacgc-3’, and CP60–215 5’-agctcaatcctgtcaag-
tgtgtaaccttc-3’. The same reverse oligonucleotide primer
3’-aagatcgggccgctggagtagtgaagatctcagtctcagta-5’, was
used for all the truncated versions of PapMV CP. The PCR
products were digested with NcoI and BamHI and inserted
into the vector pET-3d to generate the truncated clones

Media and growth conditions for synthesis of PapMV
CP27–215 in E. coli

Two different growth media were used: a rich medium
that permits large production of proteins for the biochemi-
cal characterization of the protein and one minimal
medium for 15N labeling. To overexpress in the rich media
2× YT (1.6% peptone, 1% yeast extract, and 0.5% NaCl), a
25 ml overnight preculture of E. coli strain BL21 (DE3)
RIL (Invitrogen) transformed with the pET-3d plasmid
was prepared from a single colony. A 5 ml of this preculture
was inoculated to 500 ml of the same media containing
50 µg/ml ampicillin. The protein expression was induced at
OD600 = 0.6 ± 0.1 overnight at 22 °C, 250 rpm, using 1 mM
isopropyl-β-D-galactopyranoside IPTG (Promega) and
25 µg/ml ampicillin.

For labeling with 15N, 5 ml of OD600 = 1.0 ± 0.1 precur-
ture in 2× YTamp medium was inoculated to 500 ml of the
modified M9 minimal medium [2.5 mM Betaine, 0.2 M
(Na2HPO4, 7 H2O), 0.1 M KH2PO4, 0.09 M 15NH4Cl (Cam-
bridge Isotope Labs), 0.04 M NaCl, 3.5 g/L glucose, 2 mM
MgSO4, 2 mM FeCl3, 0.1 mM CaCl2, 50 mM ZnSO4, and
0.5% w/v thiamine] containing 50 µg/ml ampicillin. The pro-
tein expression was induced at OD600 = 0.7 ± 0.1 overnight
at 22 °C, 250 rpm, using 1 mM IPTG and 25 µg/ml ampicil-
lin.

Purification of PapMV CP27–215

(a) Ni-NTA–agarose column

The whole purification procedure was performed at 4 °C.
The E. coli expression strain BL21 (DE3) RIL (Stratagene)
was transformed with the plasmid pET-3d containing
either of the different constructs pET3d-CP27–215, pET3d-
ria were cultured as described in the previous section. The
pellet was resuspended in ice-cold lysis buffer (50 mM Na
H2PO4 [pH 8.0], 300 mM NaCl, 10 mM imidazole, 40 µM
PMSF, and 0.1 mg/ml lysozyme) and bacteria were lysed by
sonication. The lysate was centrifuged twice for 30 min at
13,000 rpm to eliminate cellular debris. The supernatant
was mixed, for 4–16 h, with 1 ml of Ni-NTA–agarose
matrix (Qiagen D40724). The purification was made by
gravity-flow on a polypropylene chromatography column
(Econo-Pac Columns, Bio-Rad Laboratories). The resin
was washed with 10 bed volumes once with a first wash
buffer (lysis buffer supplemented with 20 mM imidazole),
then with the second wash buffer (lysis buffer supplemented
with 50 mM imidazole) and once with the third wash buffer
(10 mM Tris–HCl [pH 8.0] and 50 mM imidazole). The pro-
tein was incubated 30 min minimum with 2.5 bed volumes
of the elution buffer (third wash buffer supplemented with
1 M imidazole) before elution. The concentration of the
sample or the changes of buffers was done a stirred ultra-
filtration cell (Amicon, Millipore) of 3 ml with a YM10 mem-
brane (Amicon, Millipore). CP27–215 was stored in presence
of 10 mM DTT to improve protein stability and render its
concentration to 0.2 mM possible.

(b) Cation exchange and gel filtration

The whole purification procedure was made at 4 °C. After
overnight induction, cells were then harvested and resus-
pended in the cation exchange start buffer (25 mM MES, pH
6.2) containing 20 µM PMSF, 10 mM DTT, and 1 mg/ml
lysozyme (Sigma). Cells were then ruptured by at least three
passages through a French press and centrifuged twice at


140,000 rpm for 30 min at 4 °C. The supernatant was filtrated
using 0.45 μm filters. Hi prep 16/10 SP XL (GE Healthcare)
pre-equilibrated according to provider’s instructions and
was used for the first step purification. The sample was added to
the column by using a peristaltic pump (<2 ml/min) (ÄKTAbuffer
explorer pump P-910, GE Healthcare), followed by a wash
with start buffer until a 280 nm stable absorbance. The opti-
mized elution step was done by a linear gradient of 0–1 M
NaCl on 10 bed volumes where the protein was eluted at
0.3 M of NaCl. The purified protein was collected and 10 mM
DTT was added. These fractions were then pooled and puri-
fied by gel filtration. The column was calibrated with low
molecular weight marker (GE Healthcare). The Superdex 75
26/60 (GE Healthcare) pre-equilibrated with gel filtration
buffer (cation exchange start buffer supplemented by 150 mM
NaCl) was used for this second purification step. The volume
of the protein loaded in the sample loop was 1.5 ml. We add
10 mM DTT to the pooled protein fractions. Concentration
or the exchanges of the buffers was done as described before.

**SDS–PAGE and electrophoretography**

Proteins were mixed with 1/3 of the final volume of load-
ing buffer containing 5% SDS, 30% glycerol, 3 mM DTT,
and 0.01% bromophenol blue. The sample was heated at
95 °C for 10 minutes and loaded on a SDS–PAGE.

**Circular dichroism spectroscopy**

CD spectra were recorded on an Olis RSM 1000 rapid
scanner monochromator at 20 °C. For far UV CD (200–
190 nm), thermostated quartz cells of 0.1 cm path length
were used. Mean residue ellipticity values (MRW in
deg × cm² × dmol) [15] were calculated using the equation:
\[ \theta = \frac{10^2}{c \cdot l} \cdot MRW(10 \times c \times l) \]
where \( \theta \) is the ellipticity in
degrees, MRW is the average molecular weight of the resi-
dues in the protein (108 was used in this study), c is the protein
concentration in g/ml and l is the path length in cm.

**RNA transcripts and electrophoretic mobility shift assay
(EMSA)**

The RNA probe was generated by transcription in vitro
using a Ribomax Large Scale RNA Production System-T7
kit (Promega P1300) and a clone of 80nts of the 5’end of
PapMV in front of the T7 promoter. The clone was linear-
ized with EcoRI before in vitro transcription. The RNA tran-
script was purified on a G-50 Quick Spin Column (Roche
1273 965). The RNA probe was dephosphorylated using
shrimp alkaline phosphatase (Fermentas, EF0511) and
labelled with [γ-32P]ATP using T4 polynucleotide kinase
(NEB M0201S). The probe was then purified using the G-50
Quick Spin Columns as before. Labelled RNA was incu-
bated with recombinant proteins at room temperature for
60 min. We used 165 fmol of [32P] labelled RNA for each reac-
tion and various amounts of purified recombinant proteins
in 10 mM Tris–HCl, pH 8.0, and 7.5 U of RNase inhibitor

(AMersham Biosciences 27-0816-01). The final volume of the
reaction was 10 μl; 2 μl of loading dye was added to the sample
before loading onto a 5% native polyacrylamide gel. Elec-
rophoresis was performed in 0.5 × Tris–borate–EDTA
buffer for 90 min at 10 mA. The gel was dried and subjected
to autoradiography for 16 h on Kodak BioMax MS film
(Amersham Biosciences V8326886) and developed.

**Chemical cross-linking with glutaraldehyde**

Several assays with glutaraldehyde used at various concen-
trations led to the optimal condition for efficient cross linking
of the PapMV CP. We used 0.1% glutaraldehyde in 10 mM
Tris, pH 7.5, and 50 mM NaCl in a volume of 50 μl. The opti-
mal concentration of the protein used for cross linking was
150 ng/ml. After addition of glutaraldehyde, the mixture was
incubated at 20 °C for 30 min in the dark. The reaction was
stopped with 15 μl of loading dye, heated at 95 °C for 10 min
and the proteins were separated by SDS–PAGE.

**Secondary structure prediction**

Secondary structure prediction for the amino acids
sequence of PapMV CP WT involved PROSec [16], PSI-
PREP [17], Sable-2 [18], and SCRATCH [19] prediction
programs. The consensus of the various methods was deter-
mined with regard to secondary structure confidence level.

**NMR spectroscopy**

The 600 μl sample used for NMR spectroscopy was
0.1 mM CP 27–215 in 90% H2O: 10% D2O, 10 mM DTT, pH
6.2, 1× complete protease inhibitor cocktail (Roche),
0.1 mM NaN3 and 60 μM 2,2-dimethyl-2-silapentane-5-sul-
fonic acid (DSS) as the NMR chemical shift reference. The
1H–15N heteronuclear single quantum coherence spectrum
(1H–15N HSQC) were carried out at 25 °C on a Varian
Unity 600 MHz spectrometer equipped with a triple-reso-
nance cryoprobe and Z-axis pulsed-field gradient. The
acquired data consisted of 768 complex data points in
the acquisition domain and 128 complex data points in
the indirectly detected domain. The spectral width was
10,000 Hz in the 1H dimension and 1680 Hz in the 15N
dimension. The NMR spectra were processed using NMR-
Pipe [20]. Processing involved the doubling of the 15N time
domain by linear prediction, zero-filling to 2048 and 512
complex point in 1H and 15N, respectively, a 45° shifted
sine-bell apodization in the 1H dimension, and a 72° shifted
sine-bell apodization in the 15N dimension.

**Results**

**Expression and purification of PapMV CP 27–215**

The expression and the purification of CP 6–215 were
already described elsewhere [8]. In this manuscript, we also
refer to CP 6–215 as the WT protein. The expression of the
truncated versions CP_{46–215} and CP_{60–215} led to production of an insoluble protein that accumulated as inclusion bodies in \textit{E. coli}. We therefore focused our study on the protein CP_{27–215} that accumulated to high level and could be easily purified (Fig. 1). The expression of CP_{27–215} was significantly different in 2× YT\textsuperscript{amp} medium than in M9 minimal medium. The yield of purified CP_{27–215} and in 2× YT\textsuperscript{amp} medium is summarized in Table 1.

We used two purification methods. The first method is based on the affinity of a C-terminal 6× H tag on a Ni–NTA–agarose column. This purification method was done at pH 8.0, exceeding by one pH unit the predicted PI of the protein (7.06). We obtained a good enrichment using this method (Fig. 1, lane 3), but some contaminants were still present in the sample. This method was therefore not suitable for NMR analysis.

Alternatively, we used cation exchange affinity chromatography. The binding was done at pH 6.2. The protein was eluted at 0.3 M of salt. The purification procedure was completed by gel filtration on a Superdex G-75 26/60. The addition of 10 mM DTT to the sample improved its solubility and facilitated its concentration. After both steps, the purity of the protein was greater than 95% (Fig. 1, lane 4) which is suitable for NMR study.

Gel filtration and chemical cross-linking of CP_{27–215}

The expression of CP_{6–215} in \textit{E. coli} lead to formation of NLPs that can be easily identified by electron microscopy (EM) [8]. The observation of purified CP_{27–215} at the EM did not show any sign of NLPs or disk structure that are characteristic of the WT protein. Therefore, we hypothesized that CP_{27–215} is probably into a lower multimeric form. We evaluated the molecular weight of CP_{27–215} using gel filtration with a Superdex 75 10/300 and identified a unique peak (Fig. 2A) of 21.2 kDa that corresponds to a monomer. The purified protein was also submitted to a chemical cross linking in comparison with the WT recombinant protein CP_{6–215}. After cross-linking with 0.5% of glutaraldehyde, the proteins were denatured with loading dye and separated by SDS–PAGE. As previously described, CP_{6–215} was found as a large multimer that can hardly enter the polyacrylamide gel (Fig. 2B, lane 2) [8]. However, CP_{27–215} migrated as a single band and confirmed that the protein is a monomer in solution (Fig. 2B, lane 4).

Gel shift assay

The RNA binding domain of the PapMV CP was proposed to be located between amino acids 90 and 130 [21] that is included in CP_{27–215}. Therefore, CP_{27–215} should maintain its affinity for RNA as shown for CP_{6–215} [8]. We use the mobility shift assay to verify this hypothesis. Increasing amount of CP_{27–215} were incubated in a volume of 10 μl containing 165 fmol of a 32P-labelled RNA probe made from a transcript of 80 nucleotides of the 5' non-coding region of PapMV. The protein–RNA complexes were separated by an electrophoresis mobility shift assay (EMSA). The disks of CP_{6–215} interacted with the probe in a cooperative manner and induced a shift with 500 ng (22 pmol) of proteins as already shown before [8] (Fig. 3A). However, in identical experimental conditions, CP_{27–215} failed, even when as much as 1500 ng was used in the assay to induce the formation of a protein–RNA complex (Fig. 3B) which shows that this protein was unable to interact with RNA in vitro.

Secondary structure prediction and CD spectra analysis

The secondary structure of PapMV CP was predicted using four algorithms (Fig. 5), and a consensus of the four approaches was determined. The results show that the dominant secondary structure elements of the consensus are loops (54.9%) and α-helices (42.8%). To assess that the monomer CP_{27–215} has a secondary structure similar to the WT protein, we compared their CD spectra. Both spectra were done on a JASCO J-810 spectropolarimeter.

Table 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg/L)</th>
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<tbody>
<tr>
<td>Total cell lysate in 2× YT medium</td>
<td>Not determined</td>
</tr>
<tr>
<td>After Ni–NTA column</td>
<td>25</td>
</tr>
<tr>
<td>Total cell lysate in M9 minimal</td>
<td>Not determined</td>
</tr>
<tr>
<td>After cation exchange</td>
<td>5</td>
</tr>
<tr>
<td>After gel filtration</td>
<td>3</td>
</tr>
</tbody>
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were recorded in the same experimental conditions and showed to overlay upon each other (Fig. 4). This result suggests that the global secondary structure of both proteins is similar. The CD spectra of CP 27–215 and PapMV CP have two minima at 208 and 222 nm, which is typical for a protein rich in \( \alpha \)-helices and loops. This is in agreement with the secondary structure prediction, suggesting that our prediction is globally accurate.

\[ H-^{15}N \text{ HSQC spectrum of CP}_{27-215} \text{ analysis} \]

To further confirm that the monomer CP\(_{27-215}\) can be used for NMR study, we uniformly labeled the protein with \( ^{15} \text{N} \) and acquired preliminary NMR data. Several conditions were tested, and the optimal conditions for NMR were between pH 5 and 7, with 10 mM DTT at a concentration of 0.1 mM. The final sample condition for NMR analysis was at pH 6.2 to improve its solubility and stability. A 2D \( H-^{15}N \) HSQC spectrum of CP\(_{27-215}\) was acquired at 600 MHz at 25°C (Fig. 6). The good spectral dispersion (3.5 ppm) of backbone amide \( ^1H \) resonances indicates that PapMV CP is well-folded under the condition used, in agreement with the CD spectrum. Furthermore, the peak line width and signal intensity under the conditions used suggests that the mutant is monomeric in solution as expected from the chromatography results. Only few overlapping peaks are present which is
ideal for the determination of the three-dimensional protein structure determination. Finally, the $^1$H–$^{15}$N HSQC spectrum has the expected number of peaks.

**Discussion**

We have fulfilled the requirement for the preparation of a NMR sample of CP$_{27-215}$ since the protein can be produced and purified in large amount, be stable and maintained in a monomeric form in solution. The gel filtration, the chemical cross-linking and the $^1$H–$^{15}$N HSQC confirmed that the protein is a monomer. Furthermore, the CD spectra and the $^1$H–$^{15}$N HSQC showed that the protein is well folded and stable in the conditions used in this experiment. Addition of 10 mM DTT improved the solubility of the proteins probably because it inhibited the formation of non-specific S–S bridges between the subunits that could favor aggregation. The removal of the 6×H tag on CP$_{27-215}$ made the protein difficult to purify and to concentrate even in presence of DTT (data not shown). The 6×H tag appeared to be very important to improve the solubility of the protein at high concentration. We do not understand for the moment the significance of this result. The $^{13}$C–$^{15}$N labeling of the protein for 3D spectra is in process. This experiment will allow us to present for the first time the structure of the coat protein of a *Flexiviridae*.

![Fig. 4. CD spectra of CP$_{6-215}$ (grey line) and CP$_{27-215}$ (black line). Spectra were recorded at pH 7.2 in phosphate buffer at 25 °C.](image)

![Fig. 5. Secondary structure prediction of PapMV CP. We have used four prediction algorithms. Results from SCRATCH, PROFsec, PSIPREP, and Sable-2 are presented. The upper case in the consensus prediction means a high confidence level whereas the lower case means a weak confidence level. Predicted β-strand, helix, and coil or loop conformations are indicated by the letters E, H, and C (L), respectively. For the consensus predictions, uppercase letters indicate high confidence, lowercase letters indicate lower confidence, and a dot (·) indicates ambiguity. The N-terminal 26 amino acids are in bold.](image)
The N-terminus of the potexvirus CP is predicted to be exposed at the surface of the virus particle [11]. It is interesting to observe that a truncation of 26 amino acids at this extremity results in the production of a monomeric form. Consistently, the deletion of 59 amino acids at the N-terminus of pepper vein banding virus (PVBV; a potyvirus), also a member of the Flexiviridae, was crucial for the intersubunit interactions and the initiation of virus assembly [22]. A detailed analysis of the N-terminal 26 amino acid revealed a small α-helix present between amino acid 18 and 23 that could be important for the subunit interactions possibly through an electrostatic interaction of E19 and another positively charged residue. The other residues of this region form a random coil that appears very flexible because of two P in position 9 and 14 that break the structure at the extremity. It is unclear at the moment if the helix or the random coil is more important for the interaction between the protein subunits.

Also six serine residues at position 2, 4, 5, 7, 22, and 23 are found in this region. These S are putative phosphorylation targets for host kinases that could dramatically change the charge of the N-terminus. The N-terminus of the potato virus X (type member of the potexviridae) was shown to be phosphorylated by a host encoded Ser/Thr kinase. Phosphorylation of PVX CP rendered the RNA more easily translatable in vitro [23]. It is likely that phosphorylation of the N-terminus introduced repulsive charged that destabilizes subunit interactions and favored the disassembly of the virus and allow translation of the viral RNA. Several putative phosphorylation sites are also found at the N-terminus of the PapMV CP and a similar mechanism of disassembly could also apply to PapMV. The removal of the N-terminal 26 amino acids of PapMV CP probably induces a structural change of the protein that unable the formation of multimers in a similar way than phosphorylation induces disassembly of PVX.

Finally, we showed that the recombinant CP$_{27-215}$ was unable to bind RNA in vitro by EMSA in contrast to the WT protein CP$_{6-215}$. However, the putative RNA binding domain, located between amino acids 90 and 130 [21] is intact in CP$_{6-215}$. This result suggests that the multimerization of the CP is important for RNA binding, probably through a modification of the tertiary structure of the protein.

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References


