



# Production of polyclonal antiserum against Cowpea mild mottle virus coat protein and its application in virus detection

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## ABSTRACT

*Cowpea mild mottle virus* (CpMMV), the causal agent of stem necrosis disease, has drawn attention of soybean producers in recent years due to yield losses in the main producing regions of Brazil. Serological methods for viral detection require the use of an antiserum of good quality to achieve specificity and sensitivity. The entire coat protein gene of a Brazilian CpMMV isolate was cloned into a bacterial expression vector and transformed into *Escherichia coli* BL21::DE3 for *in vitro* expression. The coat protein, fused to a His-tag, was purified under denaturing conditions by affinity chromatography using a Ni-NTA resin. After renaturation, the integrity and identity of the purified recombinant protein was confirmed by SDS-Page and MALDI-ToF/ToF mass spectrometer analyses. A rabbit was immunized with increasing amounts of the recombinant protein. The specificity and sensitivity of the antiserum was demonstrated by Western blot and indirect ELISA assays. The polyclonal antisera raised against recombinant coat protein proved to be a reliable tool for CpMMV detection.

**Key words:** CpMMV, indirect ELISA, recombinant coat protein, Western blot.

Soybean (*Glycine max*) production in Brazil has increased in recent years and stands out as the country's main export crop (CONAB, 2012). Soybean stem necrosis disease has drawn the attention of soybean producers in recent years due to reduction in productivity in the main producing regions of Brazil (Almeida, 2008). The disease was first recorded in the Midwest region of Brazil in the 2000/01 soybean season and rapidly spread throughout the country in the following years (Almeida et al., 2005; Mituti & Almeida, 2006). *Cowpea mild mottle virus* (CpMMV), the causal agent of stem necrosis disease, has also been reported in Asia, West Africa, and Argentina, some of the world's main soybean producing regions, but so far not in the United States (Laguna et al., 2006; Menzel et al., 2010). Symptoms vary with the host, viral isolate and time of year but are generally seen as local chlorotic lesions, mosaic, necrosis, necrotic lesions, chlorotic rings, dwarfing and leaf distortion (Almeida et al., 2005). In soybean plants severe symptoms such as stem necrosis, necrosis of the petiole, curvature and necrosis of the shoots and plant death have been reported, although asymptomatic infections have also been reported for some soybean cultivars (Mituti & Almeida, 2006).

CpMMV belongs to the genus *Carlavirus* of the family *Betaflexiviridae*, which includes viruses with flexuous particles of 610-700 nm in length and 12-15 nm in diameter (Martelli et al., 2007). The transmission is in a non-persistent manner by whiteflies (*Bemisia tabaci*) (King

et al., 2011). The *Carlavirus* genome consists of a single-stranded positive sense RNA, 6.48-8.6 kb, including six open reading frames (ORFs), a 5' cap and a 3' poly-A tail (King et al., 2011). ORF 1 is the largest, encoding the putative methyltransferase (Mt), papain-like protease (P-Pro), helicase (Hel) and RNA-dependent RNA polymerase (RdRp) (Menzel et al., 2010). The triple gene block encodes movement proteins and consists of three overlapping ORFs (ORFs 2, 3 and 4) (Morozov & Solovyev, 2003). ORF 5 encodes a coat protein and the product of ORF 6 possesses nucleic acid-binding activity (Menzel et al., 2010).

Generally, CpMMV detection is undertaken by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) methods (Almeida et al., 2005; Laguna et al., 2006; Tavassoli et al., 2009; Menzel et al., 2010). Molecular methods such as RT-PCR are more sensitive, but their application requires the use of expensive reagents and equipment to synthesize the complementary DNA, and they are relatively complex to execute, increasing costs when indexing large numbers of samples. ELISA is more suitable for routine use and a good qualitative antibody is important to achieve specificity and sensitivity. The use of purified virus preparations is usually a time-consuming procedure and presents problems with regard to purity and the obtention of satisfactory concentrations of the final preparation. Meanwhile, recombinant proteins expressed in prokaryotic systems such as *Escherichia coli* are frequently used in research, being

stable, abundant and easily purified (Hull, 2002; Alkowni et al., 2011).

Almeida et al. (2003) developed a polyclonal antiserum from purified viral particles to detect CpMMV, but this tool is no longer available and there is currently no alternative to serological detection of the virus in Brazil. Here, we cloned, expressed and purified a complete coat protein (CP) of CpMMV, report the physicochemical properties of the recombinant protein and the production of a polyclonal antiserum against CpMMV coat protein, and demonstrate its use for detecting the virus by Western blot and indirect ELISA.

The CpMMV isolates used in this work were collected from infected soybean plants in states of Bahia, Goiás, Mato Grosso and Minas Gerais, during the years of 2009 and 2010. All isolates were maintained on soybean plants cv CD206 under greenhouse conditions. Approximately 500 ng of total RNA, extracted from soybean plants cv CD206 infected with the isolate CpMMV:BR:BA:09 using the RNeasy Plant Mini Kit (Qiagen), were reverse transcribed with SuperScript III (Invitrogen) according to the manufacturer's instructions using the forward primer 5'ACGTCTCGAGCTGGAGTCAGTGTGG3' (*Xho* I site underlined) and the reverse primer 5'ACGTGAATTCTTACTTCTTAGCGTG3' (*Eco*R I site underlined). The target cDNA was PCR-amplified using Platinum *Taq* DNA Polymerase (Invitrogen) with 40 cycles of denaturing at 94°C for 1 minute, primer annealing at 65°C for 1 minute, and extension at 72°C for 1 minute. The amplicon was purified using the Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare), digested with *Eco*RI and *Xho*I, and ligated into the pRSET-A expression vector (Invitrogen) previously digested with the same enzymes. *Escherichia coli* DH5α cells were transformed using the heat shock method (Sambrook & Russel, 2001). The clones were confirmed by PCR using the primers described above and posterior sequencing. A single clone (named pRSET-A/CP-CpMMV) was selected for expression studies.

Sequences of CpMMV and other carlaviruses were aligned using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Percent nucleotide and amino acid identities for ORF 5 (coat protein) were calculated in EMBOSS (<http://www.ebi.ac.uk/Tools/emboss/align/index.html>), using the EMBOSS needle (Global) with default settings. The software ANTHEPROT (Deleage et al., 1988) was used for the analysis of physicochemical properties based on primary structure of the CP (isolate CpMMV:BR:BA:09).

For expression of the CP, the recombinant plasmid pRSET-A/CP-CpMMV was transferred into the expression host *E. coli* strain BL21::DE3 by a heat shock procedure. A single colony was grown at 37°C, 250 rpm in LB medium containing ampicillin (100 ng/ml) until it reached an OD<sub>600</sub> of approximately 0.5, followed by the addition of IPTG to a final concentration of 1mM. Approximately 8 hours post- induction, the bacterial cells were harvested by

centrifugation (5000 *g*/10 min) and stored at -80°C. The total protein extracts were obtained according Fajardo et al. (2007). After extraction, the CP was affinity purified using a Ni-NTA column (Qiagen) under denaturing conditions, according to the manufacturer's instructions. Fractions were analysed by SDS-PAGE and visualized by Coomassie blue R250 staining (Bio-Rad) to verify the presence of the expressed protein. After dialysis the protein integrity was analyzed by SDS-PAGE and quantification was performed by Bradford's method using the Dc Protein Assay kit (Bio-Rad) according to the manufacturer's instructions.

For characterization of purified recombinant CP by MALDI-ToF/ToF mass spectrometer analyses, the Coomassie blue-stained recombinant CP band (isolate CpMMV:BR:BA:09) was recovered from the gel pieces and destained overnight with a solution of acetonitrile 50% and 25mM ammonium bicarbonate, pH 8.8. The tryptic digestion was performed according to Shevchenko et al. (2006). An additional step to remove salts and contaminants was performed using Zip Tip C18 pipette tips (Millipore), according to the manufacturer's instructions. Peptide mass mapping was performed on an UltraFlex III MALDI-ToF/ToF mass spectrometer (Burker Daltonics, USA). The peptide map was acquired in reflection positive-ion mode with delayed extraction at a mass range of 500-5000 Da. The peptide mass fingerprint (PMF) obtained was submitted to a search using the Mascot Search Engine against the NCBI nr database.

A New Zealand white rabbit (named CP2) approximately 2 months old was used for immunization. Preimmune serum from the rabbit was collected immediately before the first injection. The purified 6×His-CP was emulsified with an equal amount of Freund's incomplete adjuvant (Sigma-Aldrich) for the injections. A total of five intramuscular injections were given at weekly intervals with protein concentrations of 200, 400, 400, 800 and 800 µg for CP2. The rabbit was bled two weeks after the final injection and an additional bleeding was made two weeks later. The blood samples collected were incubated at 42°C for 1 hour and 4°C overnight to coagulate and then centrifuged at 5000 rpm for 10 minutes. The antiserum were aliquoted and stored at -20°C.

A preliminary assay to analyze the specificity of the antiserum was done by Western blot using the purified CP and the purified AC5 protein from the begomovirus *Tomato rugose mosaic virus* (ToRMV), as a negative control. Protein samples (1:10, 1:100 and 1:1000 v/v) were separated on 5 to 12% SDS-polyacrilamide gels and electroblotted onto a nitrocellulose transfer membrane (GE Healthcare) at 30 mA for 8 hours, and the remaining steps were carried out according to Hampton et al. (1990). Western blot experiments were also performed on total protein extracted from infected soybean plants. Healthy plants were used as negative control.

The polyclonal antiserum was tested by indirect ELISA in order to determine its sensitivity and the optimal

concentration to be used. Indirect ELISA was according to Clark et al. (1986), with antiserum serially diluted from 1:250 to 1:10000 and tested against soybean leaves with symptoms from the isolate CpMMV:BR:MG:09:2. Samples were considered infected when absorbance at 405 nm gave an infected/healthy ratio (I/H) of at least 2 (Sutula et al., 1986). To confirm the antiserum specificity, additional indirect ELISAs were carried out including six CpMMV isolates and *Potato virus Y* (PVY) from our collection and *Potato virus S* (PVS) kindly supplied by Prof. Antônia dos Reis Figueira from Universidade Federal de Lavras, MG.

The CpMMV:BR:BA:09 CP gene was amplified by RT-PCR from total RNA extracted from infected plants using specific primers. The PCR product, a fragment of 867 bp encompassing the entire CP gene, was purified, digested and inserted into the expression vector pRSET-A to produce the recombinant plasmid pRSET-A/CP-CpMMV. Sequence analysis confirmed the integrity of the recombinant plasmid (data not shown). The resultant plasmid was transformed into *E. coli* strain BL21::DE3 cells.

Comparing the results of antigenicity analyses and the alignment of sequences (data not shown), we could see that the antigenic regions of the CpMMV:BR:BA:09 isolate are conserved among CpMMV isolates. Moreover, low sequence identity exists between CpMMV and other carlaviruses, especially at the N-terminus, indicating probable antiserum specificity and the absence of a serological relationship between them.

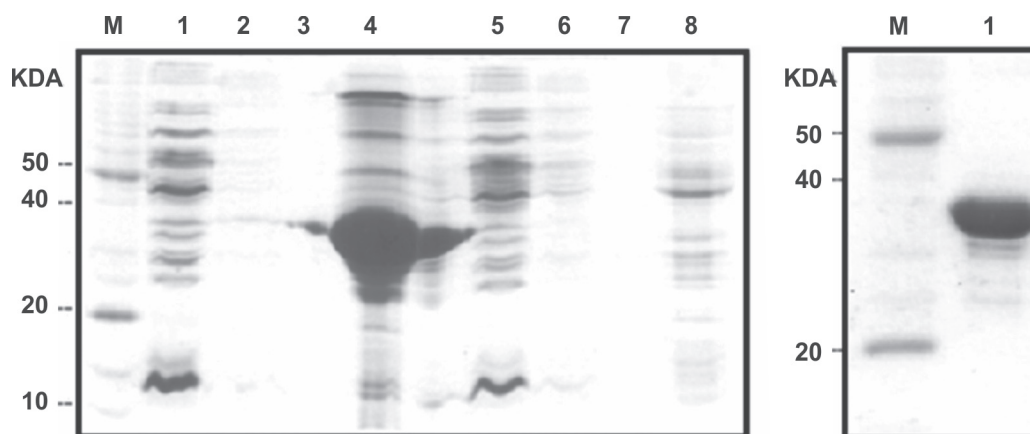
After induction with IPTG, the recombinant protein migrated in SDS-PAGE resulting in a prominent band with a molecular weight of approximately 33 kDa (the expected size of the CP plus the 6-His tag fused at the N-terminus of the protein). The band corresponding to the fusion protein was absent in the sample transformed with an empty vector (Figure 1A). The recombinant protein was successfully

extracted in a denaturant manner and purified by Ni-NTA affinity column chromatography. After refolding by dialysis, the integrity of the protein was confirmed by SDS-PAGE (Figure 1B) and the Coomassie blue-stained 33 kDa band was recovered from the gel pieces to be used in spectrophotometric analysis. The yield of the CpMMV CP fusion protein was estimated at 1.49 mg/ml, the same average yield of the carlavirus *Lily symptomless virus* (Wang et al., 2010).

In the MALDI-ToF/ToF analysis obtained from a tryptic digest of recombinant CP, the achieved peptide masses were screened against all entries in the NCBI nr database with a tolerance of  $\pm 0.5$  Da. With 38% sequence coverage, the first two protein candidates were coat proteins of different CpMMV isolates (accession numbers GU191840 and DQ444266), with scores of 103 and 102 respectively where only scores above 85 are considered significant.

Crude antiserum was tested against plant sap from young infected soybean leaves by indirect ELISA in order to determine its titer. Healthy soybean leaves were used as a negative control. Due to problems occurring with the rabbit CP1 during immunization, all tests were performed only with the antiserum obtained from rabbit CP2. The result indicates that dilutions of 1:250, 1:500, 1:1000, and 1:2000 were suitable (Table 1). An additional indirect ELISA was carried out using a 1:1000 dilution. The results showed that the antiserum raised against recombinant CP is CpMMV-specific. Cross-reaction with samples infected with either PVS or PVY was not observed (Figure 2).

Preparation of antiserum in a conventional way consists of serial injections in rabbits of viral particles purified from infected leaves (Hull, 2002). The use of purified viral particles as antigens can result in the occurrence of cross-reactions with extracts of healthy plants. The presence of antibodies to elements of the plant in the antiserum is

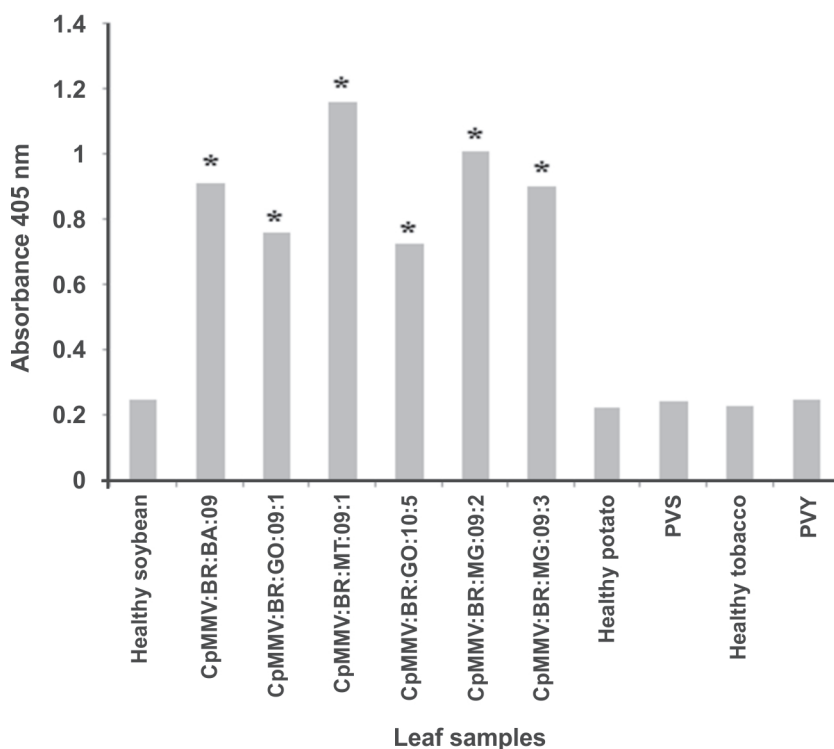


**FIGURE 1** - Electrophoretic analyses of expressed His-tagged CpMMV CP in *E. coli* BL21:DE3. (A) M, Benchmark protein ladder (Invitrogen). 1-3, Soluble protein fractions. 4, Insoluble protein fraction. 5-7, Insoluble protein fraction of *E. coli* BL21:DE3 transformed with an empty vector (pRSET-A), used as negative control. 8, Soluble portion fractions of *E. coli* BL21:DE3 transformed with an empty vector; (B) 1, Coomassie blue-stained gel of purified recombinant CpMMV-CP after dialysis.

**TABLE 1** - Titration of CpMMV CP polyclonal antiserum by indirect ELISA. Healthy and CpMMV-infected (isolate CpMMV:BR:MG:09:2) soybean leaves (cv. CD206) were tested. Goat-anti rabbit AP-conjugate was used as secondary antibody (1:2000 v/v)

Sample	Antiserum dilution					
	1:250	1:500	1:1000	1:2000	1:5000	1:10000
	<b>Absorbance 405 nm</b>					
Infected	0.734	0.599	0.507	0.438	0.329	0.306
Healthy	0.313	0.235	0.208	0.213	0.213	0.210
I/H*	2.34	2.55	2.44	2.06	1.54	1.46

\* I/H ratio represents the absorbance value ( $OD_{405}$ ) of infected samples/healthy control. A reaction was considered positive when the I/H ratio was  $>2$ .

**FIGURE 2** - Analysis of CpMMV CP polyclonal antiserum (AsCP) specificity by indirect ELISA. AsCP was used at 1:1000 dilution and goat-anti rabbit AP-conjugate was used as secondary antibody at 1:2000 dilution. Samples were considered infected when an infected/healthy ratio (I/H) of at least 2 was obtained. \*Positive samples.

due to contamination with plant components during the purification process (Almeida & Lima, 2001). Mixed infections and viral complexes are also factors that generate cross-reaction and a dubious antiserum (Fajardo et al., 2007). Another factor to consider is that some viral particles are very fragile or are at low concentration in the plant resulting in a final purified of low titer and quality (Alkowni et al., 2011). To circumvent these problems, recombinant fusion proteins expressed in prokaryotic systems are frequently used.

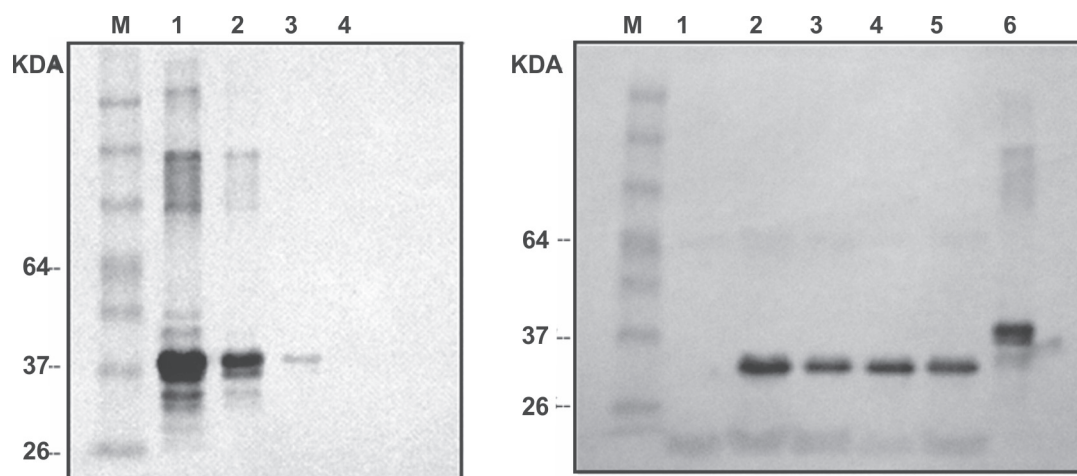
The recombinant protein used in this study was stable, easily purified and abundant. The production of specific antisera against recombinant fusion proteins has been successfully achieved for different pathosystems (Fajardo et al., 2007; Wang et al., 2010; Alkowni et al., 2011). Another advantage of this system is that the clones

with the gene of interest can be stored indefinitely at  $-80^{\circ}\text{C}$ , without the need to maintain the virus in plants.

The antisera obtained (AsCP) were able to detect the purified CP at 1:10, 1:100 and 1:1000 (v/v) dilutions in Western blot assay, and did not react with the unrelated protein used as negative control (Figure 3A). The preimmune sera from each rabbit were unable to recognize the CP in any dilution (data not shown).

The next step was to evaluate whether the antiserum were capable of recognizing the native CP from a total protein extract of soybean samples infected with CpMMV. The analyses showed that AsCP efficiently recognized the CpMMV CP from infected soybean plants and did not react with plant proteins in Western blot assays (Figure 3B). After the renaturation process, tertiary structure conformation of the recombinant protein should be similar to that of a native





**FIGURE 3** - Analysis of CpMMV CP polyclonal antiserum (AsCP) specificity by Western blot assay. **(A)** Western blot of purified recombinant CpMMV CP against AsCP at 1:1000 dilution. **M**, Prestained Benchmark protein ladder (Invitrogen). **1-3**, Purified recombinant CpMMV CP at 1:10, 1:100 and 1:1000 dilutions, respectively. **4**, ToRMV AC5 protein at 1: 100 dilution. **(B)** Western blot of total protein extracted from CpMMV-infected or healthy soybean plants, using AsCP at 1:1000 dilution. **1**, Healthy soybean plant. **2-5**, Soybean plants infected with isolates CpMMV:BR:GO:09:1, CpMMV:BR:MT:09:1, CpMMV:BR:MG:09:2 and CpMMV:BR:MG:09:3, respectively. **6**, Purified recombinant CpMMV CP, used as a positive control.

one; as a consequence, it will keep most of its antigenic determinants (Wang et al., 2010). Our study has shown that AsCP, produced against renatured CP, is capable of recognize the protein under denaturing conditions as in the SDS-PAGE and Western blot protocols.

During the purification process, besides the overexpressed CpMMV CP, some proteins constitutively expressed in *E. coli* can be brought together. The Western blot assay against the purified CP shows the reaction of the antisera with some non-specific protein (Figure 3A) that might be bacterial proteins. Since *E. coli* does not possess protein antigens serologically related with the plant, no reaction was observed when tested against plant extracts in Western blot (Figure 3B), as expected. Similar results have been observed with antiserum prepared from recombinant proteins of other viruses (Park et al., 2007).

The results presented here show that a polyclonal antiserum raised against *E. coli*-expressed and purified CP recognizes CpMMV specifically, in both Western blot and indirect ELISA systems, with a strong reaction against plant extract infected by CpMMV and no reaction with healthy plant extract or with other viral species.

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