Short Communication

Purification of recombinant *Cucumber mosaic virus* (banana isolate) coat protein by sucrose density gradient ultra-centrifugation

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Received 26 August 2020; received in revised form 3 November 2020; accepted 6 November 2020

Abstract

Cucumber mosaic virus (CMV) infected banana leaf samples were collected based on characteristic symptoms and screened by direct antigen coating immunosorbent assay and reverse transcriptase polymerase chain reaction. The expression clone of CMV coat protein was prepared using the expression vector pRSET-C and the host *Escherichia coli* BL21 (DE3)pLysS. Then, *E. coli* cells were induced by Isopropyl â-D-1-thiogalacto pyranoside (IPTG) for expression of 25 kDa recombinant CMV coat protein. After induction, the *E. coli* cells were sonicated and the coat protein was ultra-pelleted. Sucrose density gradient (10-40%) was prepared and the coat protein ultra- pellet which was dissolved in Tris-NaCl buffer of pH 8.0 was subjected to sucrose density gradient ultra-centrifugation at 26,000 rpm for 3 h at 4°C for purification. The SDS-PAGE gel profile of sucrose fractions indicated more recombinant CP in 10- 20 per cent sucrose fractions. Along with the recombinant CP, contaminant (host derived) proteins were also observed. When the coat protein ultra-pellet was dissolved in SAT buffer of pH 5.5 and subjected to sucrose gradient ultra-centrifugation as above, the 25 kDa coat protein was absent in sucrose fractions and was observed in cell pellet which indicated the insolubility of the protein in the buffer.

Key words: Buffer, *Cucumber mosaic virus*, Recombinant protein,Sucrose density gradient ultra-centrifugation.

Cucumber mosaic virus (CMV) is a major plant virus and a type member of *Cucumovirus* genus. It infects plants belonging to *Cucurbitaceae*, *Asteraceae*, *Musaceae* and *Solanaceae* families (Zitikaite and Staniulis, 2006) and is responsible for severe crop loss all over the world. The virus infects banana (*Musa* sp.) and causes infectious chlorosis/banana mosaic in tropics, especially in Kerala (Magee, 1930; Stover, 1972; KAU, 2016). The leaves of infected plants appear to have 'mosaic' symptoms and their rugosity gets often changed, making them wrinkled and malformed. The plants usually appear stunted and the reproductive phase gets retarded. CMV has a tripartite, positive sense, single stranded genome (RNA) of 8 kb, which is contained in a virion of approximately 28 nm diameter with 180 copies of coat protein (CP). The viral genome is arranged in an icosahedral symmetry with Triangulation number three (*T*=3). The genomic RNAs are further designated as RNA 1 (3.4 kb), RNA 2 (3.1 kb) and RNA 3 (2.2 kb). The first two genomic strands of CMV code for the proteins *viz*., 1a (Methyltransferase and helicase) and 2a (Replicase protein with polymerase domain) respectively. The third strand (RNA 3) expresses protein 3a, which functions in cell-to-cell movement and post- transcriptional gene silencing. A subgenomic RNA from RNA 3, often recognized as RNA 4 produces 3b, which represents nonstructural movement protein and the structural capsid protein or coat protein (Hull, 2009; Zitter and Murphy, 2009; Bujarski et al., 2019). The coat

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protein of *Cucumovirus* is multifunctional, in addition to being required for particle assembly. For example, CMV-CP is involved in the initiation of infection (Doting et al., 1977), cell to cell movement or systemic spread throughout the host (Vossen et al., 1994), and replication (Bol, 2005; Guogas et al., 2005).

Apart from detection of the virus at an early stage of infection using serological and molecular techniques, no other management measures are available for viral diseases. Antiserum production is a pre-requisite for serological detection. Earlier, this was done using partially purified virus preparations which is a cumbersome procedure.The purity and concentration of the final preparation is usually compromised due to contamination of antigens with plant proteins as well as other viral proteins in the case of mixed infection and low titre value due to the presence of inhibitory compounds. As coat protein region of CMV is sufficient to provide a reliable method for the detection of virus (Khan et al., 2012; Pandey, 2015), recombinant coat protein based antiserum is the best solution for the above mentioned problems.

The steps involved in the recombinant coat protein based antiserum production are expression of coat protein in a suitable system, purification of the expressed protein by affinity column chromatography or density gradient ultra-centrifugation followed by antiserum production in animals (rabbit/mice). In the present study, sucrose density gradient ultra-centrifugation was used to purify recombinant CMV coat protein (CP) which was expressed in pRSET-C/*E. coli* BL21 (DE3) pLysS system. Sucrose density gradient ultra-centrifugation is a standard protocol for separating small particles such as viruses and proteins from the crude (host) lysate. The dissociated CP subunits of icosahedral virus particle are capable of reassembling into virus like particles (VLPs) in sucrose density gradient (Savithri and Erickson, 1983). VLPs, being virus-like in construction, can showcase similar antigenicity of the virions and hence, make an excellent immunogen (Gopinath et al., 1994). Therefore, the present study was carried out to test whether the *E. coli* expressed CMV-CP (rCP) is capable of forming VLPs under the conditions provided so that it can be used for immunization and production of CMV specific antisera.

Virus infected samples (banana leaves) were collected from Banana Research Station, Kannara, Thrissur, Kerala, India based on characteristic symptoms (Estelitta et al., 1996; Kiranmai et al., 1996; KAU, 2016). Direct Antigen Coating-Enzyme Linked Immuno Sorbent Assay (DAC-ELISA) was carried out for immuno-diagnosis of CMV samples collected from the field. The positive samples were further confirmed by reverse transcriptase PCR amplification using forward (5'CATCGACCATGGACAAATCTGAATCAAC3') and reverse (5'CTCTCCATGGCGTTTAGTGA CTTCAGCAG3') primers (Cherian et al., 2004). A new coat protein specific primer with restriction enzyme sites was designed (Forward: 5'GGGGCTAGCATGGACAAATCTGAAT CAACC3'; Reverse: 5'CCCGGATCCTTA CTCTCCATGGCGTTTA3) and PCR amplification was carried out using high fidelity *Phusion* (*Pfu*) polymerase enzyme (Verkuil et al., 2008). The resultant amplicon and pRSET-C expression vector were subjected to digestion with Nhe1 and BamH1 enzymes and later ligated using T_4 DNA ligase at 16°C and were transformed to *E.coli* BL21(DE3)pLysS cells for expression. The recombinant BL21 cells were induced with 0.3 mM IPTG and sonicated. The supernatant collected by centrifugation at 10,000 rpm for15 min. at 4°C was then ultra-centrifuged (Beckman Coulter SW32 rotor) at 26,000 rpm for 3 h at 4°C (Fig. 1a). The ultra-pellet was resuspended in one millilitre of Tris- NaCl buffer (20 mM Tris base, 200 mM NaCl, pH 8.0) and sodium acetate (SAT) buffer (50 mM sodium acetate, 0.02% sodium thioglycolyte, pH 5.5) and later kept in end-to-end rotor at 4°C, overnight.

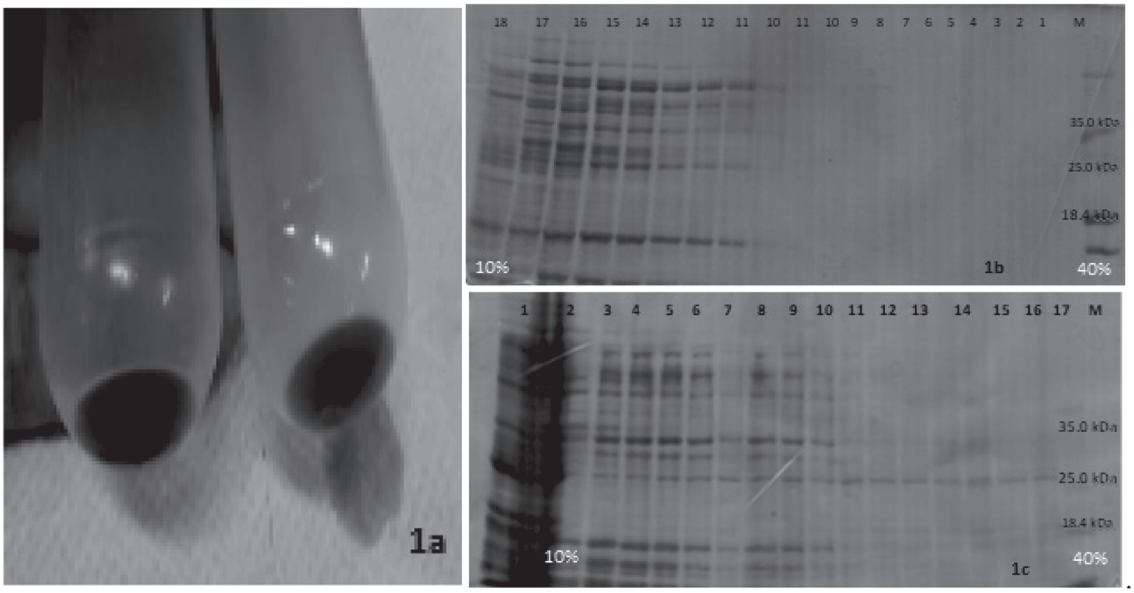

Figure 1. Purification of rCP through density gradient ultra-centrifugation (26,000 rpm, 3 h, 4°C; Beckman Coulter SW32 rotor). **a.**The ultra-pellets (*E.coli* BL21 (DE3) pLysS/pRSET-C/CMV-CP) collected after first step of ultra-centrifugation. **b.** SDS-PAGE profile of fractions collected after ultra-centrifugation of rCP dissolved in Tris- NaCl (pH 8.0) buffer, Lane 1-18 sucrose density gradient fractions ranging from 40-10%, M- Protein marker. **c.**Ultra-centrifugation of rCP dissolved in SAT buffer (pH 5.5), Lane 1: Cell pellet (collected after sonication), Lane 2- 17: Sucrose density gradient fractions ranging from 10-40%.

Sucrose solutions of 40, 30, 20 and 10 per cent concentrations dissolved in both Tris-NaCl and SAT buffers were prepared. The sucrose density gradient was prepared by gently pouring 40 per cent sucrose (8.5 ml), 30 per cent sucrose (7.5 ml), 20 per cent sucrose (6.5 ml) and 10 per cent sucrose (5.5 ml) to ultra-centrifuge tubes in the respective order. The tubes were kept at 4°C for 12 h without causing any disturbance. The ultra-pellet which was resuspended in buffers (Tris-NaCl and SAT) was centrifuged at 10,000 rpm for15 min. at 4°C and the supernatant was carefully transferred on to sucrose gradient. It was ultra-centrifuged at 26,000 rpm for 3 h at 4°C. The fractions of sucrose gradient were collected in 1.5 ml microcentrifuge tubes and electrophoresed in 12 per cent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resultant profile was observed. The fractions showing maximum concentration of CMV-CP were pooled and ultra-centrifugation was repeated. The pellet so obtained was resuspended in the respective buffer and loaded on to SDS- PAGE (Sabharwal, 2017). Western blotting was performed to confirm the presence of CMV-CP (Towbin et al., 1979).

After sucrose density gradient centrifugation of rCP in Tris-NaCl buffer (pH 8.0), eighteen fractions were collected and electrophoresed. Bands at 25 kDa between 10- 20 per cent gradient (13-18 fractions) were suspected to be that of CMV-CP (Fig. 1b). Along with CMV-CP, contaminant (host derived) proteins were also observed. The fractions were pooled, ultra-pelleted and the protein profile was observed. Sucrose density gradient centrifugation of the expressed coat protein dissolved in SAT buffer (pH 5.5) was carried out. A total of 18 fractions were collected out of which 16 gradient fractions and the cell pellet were taken for gel profiling. The concentration of 25 kDa protein in cell pellet was more, which indicated that in the buffer, CMV-CP was insoluble (Fig. 1c). Although, the protein produced was contaminated, the identity of the same was evaluated using commercially available CMV-

CP specific polyclonal antibody after which a faint band (corresponding to 25 kDa) was visualized. It was evident from the blot that the CMV coat protein purified through this method was less pure.

Purification of virus coat proteins is of paramount importance as the purified virus encoded proteins can be utilized for various studies. Scott (1963) purified native *Cucumber mosaic virus* (Cucumber isolate) particle using buffers of pH 9.0 through differential centrifugation method. He obtained approximately 80 per cent recovery of the infectious virus particle by this method. Similarly, Reddy et al. (1985) purified *Groundnut rosette virus* (genus *Umbravirus*) using ultra-centrifugation at pH 7.5 and studied the structural properties of the coat protein. Guy et al. (2015) purified nucleoprotein of the virus infecting tomato plants using ultra-centrifugation method and identified the cause of infection as *Tomato spotted wilt virus*, using mass spectrometry. In the present study, a 25 kDa band was observed in 10-20 per cent of sucrose gradient. Other *Bromoviridae* members like *Alfalfa mosaic virus* (genus *Alfamovirus*; Hull, 1970), *Tomato aspermy virus* (genus *Cucumovirus*; Habili and Francki,1974), *Hydrangea mosaic virus* (genus*Ilarvirus*; Thomas et al., 1983) and *Amazon lily mildmosaic virus* (genus *Anulavirus*, Fuji et al., 2013) coat proteins were assembled at pH ranging from 7 to 9, in 20-30 per cent sucrose density gradient which contradicted the present study. This probably indicated that the assembly of rCMV-CP in to VLPs did not take place. Gulati et al. (2015) assembled the recombinant *Sesbania mosaic virus* (family *Solemoviridae*, genus *Sobemovirus*) coat protein, an icosahedral particle of 25-30 kDa as VLPs at 20-30 per cent sucrose gradient and completely solubilised in SAT buffer. From all these studies, it could be concluded that, for each virus, the molarity and pH of buffer play an important role in the formation of virus like particles in sucrose density gradient centrifugation. The present study was aimed at producing highly pure recombinant VLPs from the Kerala isolate of CMV in *E.coli* using suitable buffer, which can be utilised for raising high quality antiserum (polyclonal) for the detection of CMV infecting banana plants. However, further investigations and standardizations are necessary to reach the end goal.

Acknowledgement

This study was supported by Dr. H. S. Savithri, INSA Senior scientist, Department of Biochemistry, Indian Institute of Sciences, Bengaluru and funded by Kerala Agricultural University, Thrissur.

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