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Short communication

Efficient purification of bromoviruses by ultrafiltration

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Abstract

Ultrafiltration using polyethersulfone-membranes was evaluated as an efficient and preferred method for purifying Cowpea Chlorotic Mottle Virus (CCMV). Cesium chloride (CsCl) ultracentrifugation and ultrafiltration protocols are described, and comparative UV-spectroscopic and electron micrograph results are presented. CCMV purified by ultrafiltration are shown to be equivalent to CCMV purified by ultracentrifugation, while reducing purification time by two days and avoiding the need for expensive capital overheads such as ultracentrifuges, rotors and toxic CsCl chemical waste.

Keywords: Bromovirus; CCMV; Virus purification; Ultracentrifugation; Ultrafiltration; Polyethersulfone membranes; Nanotechnology

1. Introduction

Bromoviruses (Broad bean mottle virus (BBMV); Brome mosaic virus (BMV); and Cowpea chlorotic mottle virus (CCMV)) are single-stranded RNA plant viruses that belong to the *Bromoviridae* family (Universal Virus Database, 2004). Their icosahedral ($T = 3$) nucleocapsids consist of 180 identical subunits (M. wt. $\sim 2 \times 10^4$ g mol^{-1}) clustered into 12 pentamers and 20 hexamers (Speir et al., 1995 and references therein). The diameter of these 'spherical' viruses varies from 25 to 28 nm.

Ultracentrifugation is a well established technique used for separating and purifying large biological molecules (e.g., Freifelder, 1982) and viruses (e.g., Bancroft et al., 1967). Ultrafiltration has been used to isolate neurotropic viruses (Smithburn and Bugher, 1953), remove viruses from tap, ground and surface water (Winona et al., 2001), and isolate viruses from seawater (Suttle et al., 1991).

This study describes methods to purify a plant virus (CCMV), and compares using CsCl ultracentrifugation to a more efficient method of ultrafiltration. The contents of this paper should be of particular interest to non-virology laboratories in need of Bromovirus samples for physical studies and nanotechnology applications.

2. Materials and methods

2.1. Inoculum, infection and leaf harvest

The inoculum used to infect plants was provided by Debbie Willits from the Mark Young and Trevor Douglas group at Montana State University and consisted of 0.1 mg ml^{-1} CCMV in an inoculum buffer (0.01 M sodium phosphate buffer, pH 6 and 0.01 M magnesium chloride).

The primary infection of plants (Cowpea California Black Eye no. 5) was done on primary leaves 8–12 days after planting and before secondary leaves emerged. The primary leaves were first coated with carborandum powder in order to abrase the leaves when the inoculum was applied. After inoculation the leaves were rinsed with water.

Secondary leaves showing infection were harvested 2–3 weeks after the initial infection and were stored at −80 °C.

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2.2. Initial purification

This procedure was done at 4 °C, required about two days and is based on Bancroft's early work (e.g., Bancroft et al., 1967). Frozen leaves were blended in buffer 1 (0.2 M sodium acetate buffer, pH 4.8 and 0.01 M disodium EDTA) to yield a thick homogenate (buffer 1 vol. was adjusted in order to get all leaves well blended, with a ratio of buffer 1 vol. to weight of leaves of approximately 2:1). The homogenate was then squeezed through three layers of cheesecloth (Fischer Scientific). This filtered homogenate was centrifuged for 15 min at 15,000 × *g*. The supernatant was collected and its volume measured. The latter was then made 10% (w/v) with PEG 8000 and stirred at 4 °C overnight. The stirred solution was then centrifuged for 10 min at 15,000 × *g*. The pellet was resuspended (with 1/10th of the buffer 1 vol. used before) with virus buffer (0.1 M sodium acetate buffer, pH 4.8 and 0.001 M disodium EDTA) and centrifuged for 10 min at 15,000 × *g*. The supernatant was collected and made 15% (w/v) with PEG 8000 and stirred at 4 °C for at least 2 h. The stirred solution was then centrifuged for 10 min at 15,000 × *g*. The pellet was carefully resuspended with a minimal volume of virus buffer, then centrifuged for 10 min at 15,000 × *g*. The final supernatant was collected and used for CsCl ultracentrifugation (see Section 2.3) and ultrafiltration (see Section 2.4).

2.3. CsCl ultracentrifugation

This procedure was done at 4 °C and required about 2 days. Approximately 2 ml of the final supernatant from Section 2.2 was loaded on 10 ml of 40% (w/w) cesium chloride solution in a Beckman Coulter ultra-clear ultracentrifuge tube and ultracentrifuged for 20 h at 248,000 × *g*. The grayish-blue-opaque viral band (located approximately half way in the tube) was extracted by using an 18 gauge needle on a 3 ml dispensable syringe. The CsCl was removed by extensive dialysis (dialyzed five times against virus buffer with a pre-washed 12,000 molecular-weight cut-off membrane (Spectrum, VWR Inc.)).

2.4. Polyethersulfone-membrane ultrafiltration

This procedure was done at 4 °C and required about 1 h. A centrifugal filter device designed for rapid concentration and purification of biological samples was required; a Centricon® Plus-20 filter from Millipore®, with a high flow Biomax® ultrafiltration membrane (polyethersulfone) with a cut-off of 300,000 nominal molecular weight limit (NMWL) was used. This device is made of two parts: the sample filter upper cup with the membrane where the initial sample is poured and recuperated and the lower filtrate collection tube. The Centricon® Plus-20 is compatible with any bench-top, swinging-bucket centrifuge that can accommodate 50 ml centrifuge tubes (29–31 mm, o.d.). The final supernatant from Section 2.2 was loaded into the upper cup (maximum capacity 19 ml) of a 300,000 NMWL Centricon® Plus-20 and centrifuged for 5 min at 4000 × *g*. To purify, approximately 19 ml of virus buffer was added to the retained CCMV sample and centrifuged for 1 min at 4000 × *g*. This last step was repeated three times.

2.5. UV-spectroscopy

Virus concentration and purity were determined by measuring the UV-absorbance at 260 nm and 280 nm in a 1 cm quartz cell with a HP 8453 spectrometer. The reference spectra of the virus buffer were subtracted from the UV-spectra for CCMV in virus buffer.

2.6. Electron microscopy

The presence of intact CCMV virus was confirmed by electron microscopy (EM). All electron micrographs were obtained from CCMV samples in virus buffer in the concentration range of 0.1–0.5 mg ml^{-1}. Carbon-coated, parlodion support films mounted on copper grids were made hydrophilic immediately before use by high-voltage, alternating current glow-discharge. 20 μl CCMV samples were applied directly onto grids and allowed to adhere for 2.5 min. Grids were rinsed with three drops of distilled water, negatively stained with 1% uranyl acetate for 30 s and air-dried. Specimens were examined in a Hitachi H-7000 electron microscope at an accelerating voltage of 75 kV. Images were recorded on Kodak electron microscope film 4489.

3. Results

3.1. CsCl ultracentrifugation

The CCMV UV-spectrum (Fig. 1a) is typical of a viral solution where the 260 nm absorbance (Abs_{260}) is mainly due to the nucleic acid (in this case RNA) and the 280 nm absorbance (Abs_{280}) is mainly due to the capsid protein.

The ratio Abs_{260}/Abs_{280} is used to determine viral sample purity, and for CCMV a ratio of 1.5–1.7 is a good sample quality. For the CCMV sample purified by CsCl ultracentrifugation the Abs_{260}/Abs_{280} ratio is 1.6 (Fig. 1a). The electron micrograph of the same sample shows intact, spherical CCMV virions (Fig. 1b).

3.2. Polyethersulfone-membrane ultrafiltration

For the CCMV sample purified by ultrafiltration the Abs_{260}/Abs_{280} ratio is 1.6 (Fig. 2a). The electron micrograph of the same sample shows intact, spherical CCMV virions (Fig. 2b).

Since the capsid protein in BBMV lacks trytophan, its Abs_{280} is significantly less than the capsid protein for CCMV and for BMV. Our calculated (Gill and von Hippel, 1989) protein extinction (ε_{280}) coefficients of 23590 M^{-1} cm^{-1} (CCMV), 23470 M^{-1} cm^{-1} (BMV) and 5240 M^{-1} cm^{-1} (BBMV) show that the Abs_{260}/Abs_{280} ratio will be similar

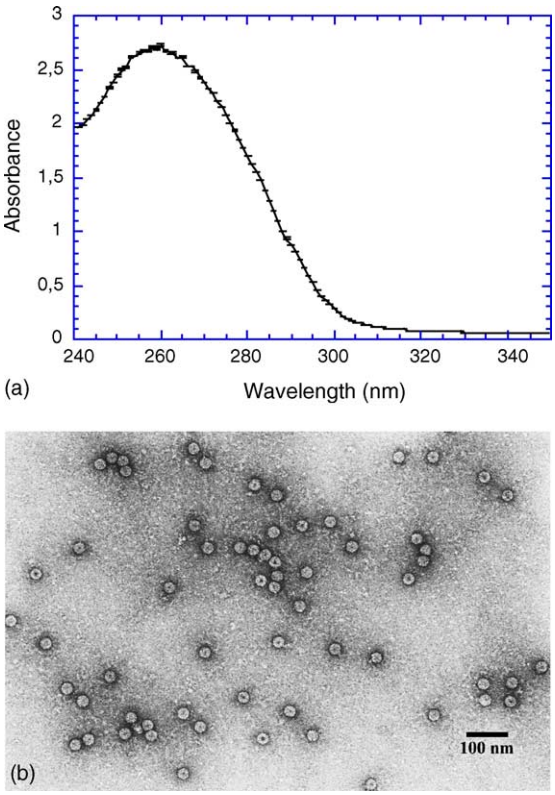

Fig. 1. (a) UV-absorbance spectrum of CCMV in virus buffer after purification by CsCl ultracentrifugation and dialysis. Using 1 OD_{260} = 0.17 mg ml^{-1} and Abs_{260} = 2.7 we calculate the CCMV concentration to be 0.46 mg ml^{-1}. We have noticed that the literature (experimental) values for converting optical density at 260 nm to CCMV concentration vary between 1 OD_{260} = 0.17 and 0.2 mg ml^{-1}. Using our calculated extinction (ε_{260}) coefficient for CCMV, our calculated conversion is 1 OD_{260} = 0.15 mg ml^{-1}. In order to be consistent with the literature (empirical) values we use the conversion 1 OD_{260} = 0.17 mg ml^{-1} for a 1 cm cuvette pathlength. Note that $Abs_{260} = \varepsilon_{260}$ C L, where C is the concentration and L is the cuvette pathlength. (b) Electron micrograph of CCMV sample after purification by CsCl ultracentrifugation and dialysis.

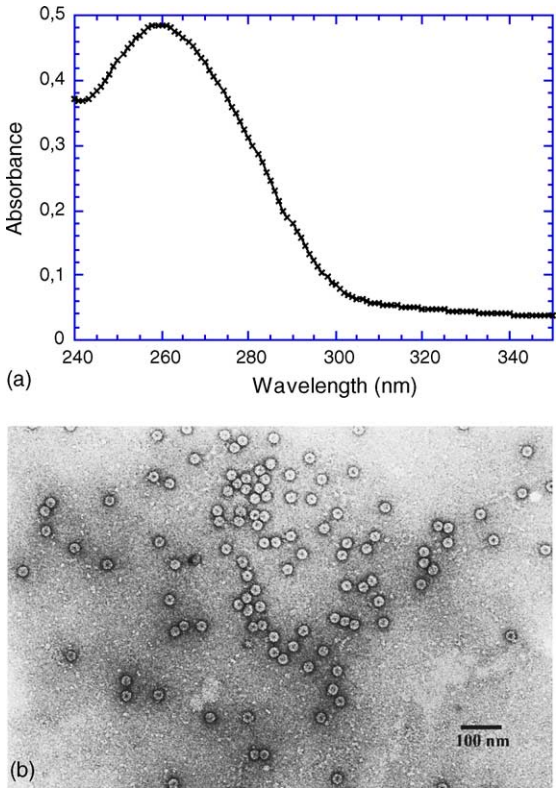

Fig. 2. (a) UV-absorbance spectrum of CCMV in virus buffer after purification by ultrafiltration with a 300,000 NMWL Centricon® Plus-20. Using 1 OD_{260} = 0.17 mg ml^{-1} and Abs_{260} = 0.48 we calculate the CCMV concentration to be 0.08 mg ml^{-1}. (b). Electron micrograph of CCMV sample after purification by ultrafiltration with a 300,000 NMWL Centricon® Plus-20.

for CCMV and for BMV (with good purity samples within a range of 1.5–1.7), whereas a pure BBMV sample must have a much higher Abs_{260}/Abs_{280} ratio (we estimate about six).

4. Discussion

The results presented here are for one set of parallel CsCl ultracentrifugation and ultrafiltration experiments. In all our CCMV purifications using CsCl ultracentrifugation or ultrafiltration we have measured Abs_{260}/Abs_{280} ratios within the acceptable range of 1.5–1.7 for pure CCMV.

The reproducibility of these purification procedures is good to two significant figures (Abs_{260}/Abs_{280} ratios and CCMV concentrations (mg ml^{-1})). However, we have observed that the CsCl ultracentrifugation method yields slightly better quality samples. For example, in another (parallel) CsCl ultracentrifugation and ultrafiltration procedure we have Abs_{260}/Abs_{280} ratios of 1.44 (after initial purification); 1.56 (after ultrafiltration); and 1.62 (after CsCl ultracentrifugation and dialysis).

Since CCMV yields (mg CCMV per gram plant leaf) depend on the strength of the inoculum, type of light source, duration of light exposure, health of the plant, etc. (e.g., Bancroft et al., 1967), we have not systematically determined total viral yields. However, we have found that the CsCl ultracentrifugation and subsequent (required) extensive dialysis gives lower yields of purified CCMV than our ultrafiltration method.

It is important to note that we find no difference in the infectivity of CCMV purified by ultrafiltration to that obtained by ultracentrifugation. For example, leaves infected by CCMV purified by ultrafiltration yielded 0.23 mg CCMV per gram plant leaf.

In developing and testing these purification protocols we also used an ultrafiltration membrane with a cut-off of 100,000 NMWL and found the CCMV sample was no purer than the CCMV sample after initial purification (see Section 2.2).

We hope this paper will be a useful resource for purifying Bromoviruses using CsCl ultracentrifugation and ultrafiltration. In addition ultrafiltration should be useful for purifying viruses in general, and as more ultrafiltration membranes are made commercially available (500,000 NMWL, etc.), they should prove very useful in purifying larger viruses.

We have found ultrafiltration to be an efficient one-step procedure that purifies CCMV, concentrates the CCMV sample to the desired concentration and allows for the quick changing of a buffer for different experiments. Our CCMV samples are currently being used in atomic force microscopy (AFM), virus re-assembly, and osmotic stress studies.

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References

Bancroft, J.B., Hills, G.J., Markham, R., 1967. A study of the self-assembly process in a small, spherical virus formation of organized structures from protein subunits in vitro. Virology 31, 354–379.

Freifelder, D., 1982. Physical Biochemistry, second ed. W.H. Freeman and Company, New York, chapter 11.

Gill, S.C., von Hippel, P.H., 1989. Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. 182, 319–326.

Smithburn, K.C., Bugher, J.C., 1953. Ultrafiltration of recently isolated neurotropic viruses. J. Bacteriol. 66 (2), 173–177.

Speir, J.A., Munshi, S., Wang, G., Baker, T.S., Johnson, J.E., 1995. Structures of the native and swollen forms of cowpea chlorotic mottle virus determined by X-ray crystallography and cryo-electron microscopy. Structure 3, 63–78.

Suttle, C.A., Chan, A.M., Cottrell, M.T., 1991. Use of ultrafiltration to isolate viruses from seawater which are pathogens of marine phytoplankton. Appl. Environ. Microbiol. 57 (3), 721–726.

Winona, L.J., Ommani, A.W., Olszewski, J., Nuzzo, J.B., Oshima, K.H., 2001. Efficient and predictable recovery of viruses from water by small scale ultrafiltration systems. Canad. J. Microbiol. 47 (11), 1033–1041.

Universal Virus Database, 2004. http://www.ncbi.nlm.nih.gov/ICTVdb.