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PURIFICATION AND ELECTRON MICROSCOPY OF SOME ISOLATES OF BLACKEYE COWPEA MOSAIC AND COWPEA APHID-BORNE MOSAIC POTYVIRUSES

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Abstract

Two isolates of blackeye cowpea mosaic (BlCMV) and two of cowpea aphid-borne mosaic (CABMV) potyviruses were purified by three purification procedures. Variable virus yields of each isolate of each virus were obtained with different virus-host combinations and purification procedures. Highest average virus yields of 4.6 μg and 3.4 μg per 50 g of infected tobacco leaf tissue were obtained from CABMV isolate RN-7C and BlCMV isolate PU-7B. Method-3 was most effective for purification of both viruses.

Carbon tetrachloride when used in combination with chloroform as a clarifying agent improved purification of the BlCMV isolates, but was harmful to CABMV isolates. Borate buffer was superior than phosphate for purification of both viruses. Addition of EDTA and Triton X-100 in extraction and resuspension buffers circumvented virion aggregation.

Leaf dip or purified virus preparations of BlCMV or CABMV isolates, examined under the electron microscope, contained elongated flexuous particles of modal length of 742 nm and 725 nm for BlCMV and CABMV, respectively, and confirmed particle morphology of potyvirus group.

Introduction

Potyviruses are difficult to purify because of their tendencies towards irreversible aggregation during extraction and concentration with consequent virus loss during low speed centrifugation. Several protocols have been described for the purification of blackeye cowpea mosaic (BlCMV) and cowpea aphidborne mosaic (CABMV) potyviruses (Lima *et al.*, 1979; Purcifull & Gonsalves., 1985; Zaho *et al.*, 1991; Ross, 1967, Bock & Conti, 1974). During a study of cowpea germplasm evaluations for detection and identification of seed-borne potyviruses, several isolates of BlCMV and CABMV were identified (Bashir, 1992). Four selected isolates, 2 of BlCMV and 2 of CABMV were compared for purification and electron microscopy. The specific objectives of this study were (a) to standardize the purification procedure for BlCMV and CABMV and (b) to examine particle morphology and to measure virion size for the identity of each virus isolate. This paper presents the results of purification and electron microscopy of four isolates of BlCMV and CABMV potryviruses.

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Materials and Methods

Source of Virus Isolates: Two isolates of BICMV (PI-26B and PU-7B) and 2 of CABMV (RN-7C and PI-23B) derived from cowpea germplasm in another study were selected for purification of virus and electron microscopy. The identity of each isolate of BICMV and CABMV was confirmed by double antibody sandwitch enzyme-linked immunosorbent assay (DAS-ELISA) using polyclonal antisera to BICMV and CABMV (Bashir, 1992).

Virus Propagation: Two host species tobacco (*Nicotiana benthemiana*) and cowpea (*Vigna unguiculata*) were used for virus propagation. Plants were raised under greenhouse conditions and mechanically inoculated with respective isolate of each virus. Inoculations were made by rubbing carborundum-dusted fully-expanded leaves with extracts from plants infected by individual isolate of each virus. Inoculated plants were maintained in insect-free greenhouse conditions at 22-25°C; 14 h photoperiod. Infected leaves from inoculated plants were harvested after two weeks of inoculation and kept at 4°C for 24 h before purification was started.

Virus Purification: Three methods were tried for purification of isolates of BICMV and CABMV. Initially CABMV isolate RN-7C was purified by Method-1, as described by Bock (1973) with some modifications, to produce antiserum against this isolate (Bashir, 1992). Two other methods were subsequently developed here called, Method-2 and Method-3, and are described below. Fifty infected leaf tissue was used each time for purification. Each method was repeated twice with each virus-host combination and the average virus yield was calculated.

Method 1

Two weeks after inoculation systemically infected leaves (50 g) were harvested and homogenized in 0.5 M phosphate buffer, pH 7.0 (1:5 w/v) containing 0.72 % 2-mercaptoacetic acid, 6% urea, 0.2% Triton X-100, 0.2% antifoam B, 0.01 M sodium diethyldithiocarbamate (Na-DIECA) and 0.001 M MgCl2. The slurry was clarified with chloroform (1 ml/ g tissue) and the aqueous phase was subjected to a cycle of differential centrifugation (118,000 g and 7,000 g). The pellets were resuspended in 0.5 M phosphate buffer, pH 7.0, containing Triton X-100 and Na-DIECA, clarified by low speed centrifugation, and layered on 10-40% sucrose density gradients prepared in 0.5 M phosphate buffer. Virus fractions were collected by running the gradient columns on ISCO fractionator and concentrated by ultracentrifugation (118,000 g). The final pellet was suspended in 0.01 M phosphate buffer, pH 7.0. Virus concentration was estimated spectrophotometrically with an extinction coefficient of 2.4 (1 mg/ml) cm^1 at 260 nm after correction for light scattering. An A260/A280 ratio of 1.2 was used as an index of viral purity (Lima *et al.*, 1979).

Method 2

This method of purification was basically the same as described by Hampton *et al.*, (1992). The virus was extracted from prechilled systemically infected leaves homog-

enized with cold 0.2 M borate buffer, pH 8.0, containing 0.5% mercaptoethanol, 1mM EDTA, 1% Triton X-100, 1% antifoam B, and 20% (v/v) chloroform. The last three reagents were added just before use of the buffer. Homogenized tissue was subjected to low speed centrifugation (3,000 g). The supernatant was filtered through glass wool, precipitated by stirring for 45 min., with 6% polyethylene glycol (PEG), and centrifuged, 10 min, 16,000 g. The resulting pellet was resuspended in cold (0.01 M borate buffer, pH 8.0, containing 0,05% mercaptoethanol, 1mM EDTA, and 1% Triton X-100) called buffer B. The suspension was clarified by centrifugation, 10 min., 3,000 g filtered through glass wool, centrifuged, 90 min., 118,000 g and the pellet was suspended in buffer B. The suspension was layered on 10-40% sucrose density gradients (prepared in buffer B), and centrifuged, 150 min., 104,000 g. Bands associated with virions were manually collected, diluted 1:1 with buffer B and centrifuged, 90 min., 118,000 g. The final pellet was suspended in 0.01 M borate buffer, pH 8.0, without any additives, and the virus concentration was estimated spectrophotometrically.

Method 3

In the course of developing of this method, elements of several published methods were evaluated including Lima *et al.*, (1979); Taiwo *et al.*, (1982); and Zaho *et al.*, (1991). None of several efforts resulted in success, primarily because of virion aggregation. The protocol outlined below was essentially a modification of Method 2 and worked satisfactory for purification of BlCMV and with some modifications also for CABMV.

Systemically infected leaves were harvested and homogenized in 0.2M borate buffer, pH 8.0, containing 0.75% 2 mercaptoethanol, 0.001 M EDTA, and 1% Triton X-100 (called extraction buffer EB). The plant extract was clarified with chloroform (20% volume of EB) and carbon tetrachloride (10% volume of EB), precipitated with 5% polyethylene glycol and resuspended. The suspension underlined with 40% sucrose cushion was subjected to ultracentrifugation, 90 min., 118,000 g. Resulting pellets were resuspended at 4°C overnight in 0.01 M borate buffer, pH 8.0 containing, 2 ME, EDTA and Triton X-100. The suspension was run onto 10-40% sucrose density. Virus bands were manually collected and precipitated. The final pellet was resuspended in 0.01 M borate buffer pH 8.0, and the virus concentration was estimated by the procedure as mentioned before.

Electron microscopy: Two isolates PI-26B (BlCMV) and RN-7C (CABMV) were used for electron microscopy. Leaf dip preparations or purified virus of each isolate were applied to carbon-stabilized formvar-coated copper grids, negatively stained with 2% ammonium molybdate, pH 7.0 and examined by a Phillips Model CM 12 transmission electron microscope and photographed. About 100 particles each of CABMV isolate RN-7C and BlCMV isolate PI-26B were measured. The virion length occurring most frequently was treated as its modal length (Walkey, 1985). The data were assembled and analyzed as a histogram as outlined by Tomlinson (1964).

This study was conducted in Virology Laboratory, Oregon State University, Corvallis, Oregon, U.S.A.

Results

Virus Propagation and Purification: Variable yield of isolates of BlCMV and CABMV virions was observed with different combinations of host species, virus isolates, and purification methods (Table 1). In general tobacco (*N. benthemianan*) was suitable host for purification of both BlCMV and CABMV isolates. A great variation in virus yield in each virus-host combination was observed. In case of BlCMV highest virus yield (3.4 μg) was obtained with isolate PU-7B with Method-3, when purified from tobacco. In case of BlCMV a reasonable virus yield was obtained with Method-3, irrespective of host species. The Method-1 and Method-2 did not prove effective to get better virus yield of BlCMV isolates.

In case of CABMV the highest virus yield (4.6 μg) was obtained with isolate RN-7C with Method-3 when purified from Tobacco. The Method-1 did not work well, however Methods 2 and 3 gave reasonable virus yield. The Method-3 was the most effective and the virus yield was significantly increased over the other two methods with each isolate when purified either from tobacco or from cowpea leaves. It was interesting to note a variation in virus yields not only between the two viruses, but also among the isolates of the same virus with different host and purification methods.

Among three purification methods tried, Method-2 and Method-3 worked well for CABMV isolates. Only Method-3 was suitable for BlCMV isolates. The main difference in the two methods was the addition of CCl4 and chloroform in Method-3 and a lower concentration of PEG in Method-3 (5%) than in Method-2 (6%). The virions of most CABMV isolates were disintegrated by CCl4, whereas BlCMV isolate PI-26B was stable in the presence of CCl4.

Electron microscopy: Flexuous rod-shaped particles were consistently found in leaf dip preparations prepared either from infected cowpea/tobacco leaves with isolates of BlCMV or CABMV or purified virus preparations. Occasionally, virus aggregates were also found in purified preparations. The histograms in Fig.1 present the length of particle distribution of CABMV isolate RN-7C and BlCMV isolate PI-26B. The figures show a maximum particle length between 700 to 800 nm for these isolates. The modal length for CABMV isolate RN-7C was 742 μm and 725 μm for BlCMV isolate PI-26B.

Discussion

The choice of a host plant for virus propagation is often critical and it varies with different viruses and virus strains. The tobacco species *N. benthemiana* gave higher virus yields of isolates of BlCMV and CABMV than cowpea. Suitability of tobacco species for virus purification and particularly for potyviruses has been reported (Hollings & Brunt, 1981; Purcifull & Gonsalves, 1985). Virus concentration in the propagation host is also influenced by both the age of the host and environmental conditions. Different strains of a virus often require different periods of time to reach maximum concentration in systemically infected tissue (Steere, 1959).

The average absorbance A260/A280 (nucleic acid/protein, absorbing regions) ratios for the purified isolates of BlCMV and CABMV were recorded between 0.720 to 1.312 (Table 1). In some cases the values were below than the reported values of 1.2

Table 1. Average virus yields and absorbance (A260/280) ratios of BlCMV and CABMV isolates purified from tobacco and cowpea by purification methods No. 1, 2 and 3.

Isolate	Host species	Method	Average virus yield (mg/50g)	Average A260/280) ratios
BlCMV isolates:				
PI-26B	Tobacco	1	0.5211	1.110
		2	0.721	0.582
		3	2.126	1.120
PI-26B	Cowpea	1	0.326	0.720
		2	0.412	1.002
		3	1.416	1.190
PU-7B	Tobacco	1	0.289	1.024
		2	0.510	0.876
		3	3.416	1.207
PU-7B	Cowpea	1	0.348	0.926
		2	0.390	1.016
		3	3.120	1.096
CABMV isolates:				
RN-7C	Tobacco	1	0.516	1.112
		2	2.240	1.320
		3	3.632	1.068
RN-7C	Cowpea	1	0.456	1.230
		2	1.446	0.851
		3	1.360	1.312
PI-23C	Tobacco	1	0.496	0.988
		2	1.980	1.102
		3	2.240	1.105
PI-23C	Cowpea	1	0.260	1.101
		2	1.682	1.208
		3	3.448	1.176

Average of two replications.

for BlCMV and CABMV (Lima *et al.*, 1979) and other potyviruses (Hollings & Brunt, 1981). Slight variation in absorbance ratios, than the reported values for individual virus and virus isolates are possible and depends upon the accuracy of purification methods.

Aggregation of virus particle and virus and host components during purification have been identified as limiting factors in obtaining higher yields especially in potyviruses (Barnett & Alper, 1977; Hiebert & MacDonald, 1973; Shepherd & Pound, 1960; van Oosten, 1972; Hollings & Brunt, 1981). However, losses associated with low speed centrifugation due to aggregation of virus particles, were reduced by the addition

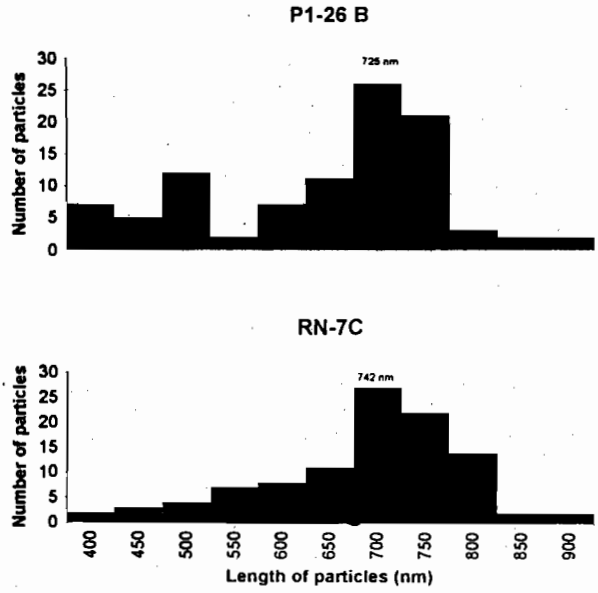

Fig.1. Histograms of particle lengths of BlCMV isolate "PI-26B" with modal length of 725 nm and of CABMV isolate "RN-7C" with modal length of 742 nm.

of EDTA and Triton X-100 to grinding and subsequent resuspension buffers. The effectiveness of EDTA and Triton X-100 in reducing virus aggregation during virus purification has been reported for potyviruses (Taiwo *et al.*, 1982; Hollings & Brunt, 1981).

Different potyviruses show widely different tolerance to various clarifying reagents. The most widely used clarifying reagent for potyviruses has been chloroform (Hollings & Brunt, 1981). Carbon tetrachloride has also been used at a concentration of 12.5 - 25% to clarify bean yellow mosaic virus (BYMV), clover yellow vein virus (CYVV), plumb pox virus, and BlCMV potyviruses (Hollings & Brunt, 1981; Zhao *et al.*, 1991). The purification protocol was improved for BlCMV isolates and the virus was stable when CCl4 was used in combination with chloroform in Method-3 but it did not work for CABMV isolates and disintegration of virus particles was observed. The use of CCl4 in combination with chloroform to purify BlCMV (Zaho *et al.*, 1991) and BYMV, bearded iris mosaic, dasheen onion and yellow dwarf potyviruses (Hollings & Brunt, 1981) has been reported with good results and we also obtained the same results with BlCMV isolates. In the present study borate buffer was found better than phosphate buffer for the purification of BlCMV and CABMV isolates. The superiority of borate buffer over phosphate has previously been reported by Shepherd & Pound (1960) with turnip mosaic virus and by Tomlinson (1964) with lettuce mosaic virus.

The modal length of 742 nm of CABMV isolate RN-7C and 725 nm for BlCMV isolate PI-26B virions was recorded, which is in agreement with the published results reported for CABMV and BlCMV (Bock, 1973; Lovisolo & Conti, 1966; Edwardson & Christie, 1986).

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