Purification, Partial Characterization, and Serology of Blackeye Cowpea Mosaic Virus

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ABSTRACT

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Blackeye cowpea mosaic virus (BlCMV) was increased in cowpea, *Vigna unguiculata* 'Knuckle Purple Hull', and infected leaves were used for purification of virus and cytoplasmic inclusion bodies. Either *n*-butanol or a combination of chloroform and carbon tetrachloride was used in the clarification process. Polyacrylamide gel electrophoresis of sodium dodecyl sulfate (SDS)-dissociated inclusions and virions revealed that the inclusions consisted of a single protein estimated to have a molecular weight (MW) of about 70,000 whereas freshly purified BlCMV consisted of a main protein component with a MW of 34,000 and two smaller proteins with MWs of 29,000 and 27,000. Purified BlCMV had a A_{260}/A_{280} ratio of 1.2 and a modal length of 753 nm. Freshly purified BlCMV preparations showed a single sedimenting peak with $s_{20} = 157–159S$. Storage of purified BlCMV at 4 C for more than 30 days resulted in a reduction of the virion S value, a reduction in the molecular weight of the capsid protein, and a partial loss of antigenic determinants. The purified BlCMV cytoplasmic inclusions had absorption spectra characteristic for proteins. Antisera reactive in SDS-immunodiffusion were obtained against untreated virions, pyrrolidine-dissociated coat protein, and untreated BlCMV cytoplasmic inclusions. Double immunodiffusion tests (with SDS-treated antigens and antiserum to BlCMV) showed that the BlCMV is serologically unrelated to six potyviruses and serologically related to, but distinct from, the following potyviruses: bean common mosaic (BCMV), bean yellow mosaic, cowpea aphid-borne mosaic (CAMV), dasheen mosaic, lettuce mosaic, tobacco etch, potato Y, soybean mosaic, and watermelon mosaic-2. The intragel cross-absorption technique also was used to distinguish BlCMV from CAMV and BCMV. Five of 20 cowpea cultivars were resistant to BlCMV, whereas all 20 were susceptible to CAMV.

Virus diseases are considered to be a major limiting factor affecting the production of cowpeas (*Vigna unguiculata* [L.] Walp.) in several countries (5,6,11,16,17,22,34). Several viruses infect cowpea, and many of them are seed transmitted. The most important seed-borne virus of cowpea in the southeastern USA is an aphid-transmitted filamentous virus approximately 750 nm long (11,29,34,35). It was first isolated in Florida by Anderson (2), who designated it "blackeye cowpea mosaic virus" (BlCMV) (3).

The research being reported here was undertaken to characterize BlCMV and to determine its relationship to other potyviruses. Methods for virus and inclusion purification, as well as certain physical, biological, and immunochemical properties of BlCMV are described here for the first time. An abstract describing part of this research has been published (18).

MATERIALS AND METHODS

Source of the virus isolate. The blackeye cowpea mosaic virus used in this study was isolated from infected seeds of cowpea(*V. unguiculata* 'Knuckle Purple Hull') harvested from a field in Gainesville, Florida. The virus was transmitted by aphids from infected cowpea plants grown from infected seeds to noninfected Knuckle Purple Hull plants. Two aphids (*Myzus persicae*, Sulz.) were used per test plant and each aphid was allowed a 30 to 60 sec access period. A single test plant showing typical mosaic was assayed by leaf-dip electron microscopy for the presence of rod-shaped virus particles and used as the initial source of inoculum for virus propagation. The virus was mechanically transmitted from the selected infected plant to healthy Knuckle Purple Hull seedlings, where it was increased for virus and inclusion purification, and other studies.

Virus and inclusion purification. Blackeye cowpea mosaic virus was propagated in either *V. unguiculata* or *Nicotiana benthamiana* Domin, and systemically infected leaves were used for virus and inclusion purification. Primary leaves of 5- to 7-day-old cowpea seedlings were inoculated with BlCMV obtained by grinding infected leaf tissue in potassium phosphate buffer (PB) (2 ml of 0.05 M PB, pH 7.5, per gram of tissue). The first trifoliolate leaves showing typical mosaic were collected 15–18 days later and subjected to the following purification procedures. All *g* forces given are maximum values.

Butanol clarification method. Two hundred to 400 g of leaf tissue were homogenized in a blender with 0.5 M PB, pH 7.5, containing 0.5% or 1.0% Na_2SO_3 (2 ml buffer per gram of tissue). The resulting extract was filtered through a double layer of cheesecloth and *n*-

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butanol was added to make a final concentration of 8% (v/v). This mixture was stirred overnight at 4 C and the coagulated green debris obtained was removed by a low speed centrifugation at 11,700 *g* for 10 min. Virions were precipitated from the supernatant by the addition of 6–8% (w/v) of polyethylene glycol MW 6000 (PEG) followed by stirring for 60 min at 4 C. The precipitated virions were collected by centrifugation at 13,200 *g* for 10 min. The resulting pellet was resuspended in 0.02 M PB, pH 8.2, containing 0.1% 2-mercaptoethanol (2-ME) (v/v) and the virus was separated from the host components by equilibrium density gradient centrifugation (150,000 *g* for 16–18 hr in a Beckman SW 50.1 rotor) in 30% cesium chloride (CsCl) prepared in the same buffer. The virus zone, located at 12–15 mm from the bottom of the tube, was collected dropwise through a hole punched in the bottom of the tube and diluted with 0.02 M PB, pH 8.2, containing 0.1% 2-ME. The virus preparation was clarified by centrifugation at 11,700 *g* for 10 min and reconcentrated by centrifugation at 120,000 *g* for 90 min. The final pellet was resuspended in 0.02 M Tris buffer, pH 8.2, and the virus concentration was determined spectrophotometrically using an extinction coefficient of $E^{0.1\ o/o}_{261\ nm} = 2.4$, the value determined for tobacco etch virus (24). The A_{260} and A_{280} values were corrected for light scattering.

Chloroform-carbon tetrachloride clarification method. This clarification process was used when it was desirable to purify both the virus and inclusions from the same batch of tissue. Systemically infected tissue (200–400 g) was homogenized with a Sorvall Omnimixer in a solution containing 1.30 ml of 0.5 M PB (pH 7.5), 0.35 ml of chloroform, 0.35 ml of carbon tetrachloride, and 5.0 mg of Na_2SO_3 per gram of tissue. The homogenized mixture was centrifuged at 4,000 *g* for 5 min and the pellet containing the organic solvents was discarded. The aqueous phase was centrifuged at 13,200 *g* for 15 min to precipitate the virus-induced inclusions. The virus in the supernatant was precipitated with PEG, purified as described above, and the pellet containing the inclusions was resuspended in 0.05 M PB, pH 8.2, and 0.1% 2-ME. The inclusion suspension was homogenized in a Sorvall Omnimixer for two min and Triton X-100 was added to a final concentration of 5% (v/v). After being stirred for 1 hr at 4 C this mixture was centrifuged at 27,000 *g* for 15 min to pellet the inclusions. The pellet was resuspended in 10–20 ml of 0.02 M PB, pH 8.2, containing 0.1% 2-ME, and homogenized for 30 sec. The inclusions were pelleted again by centrifugation at 27,000 *g* for 15 min. The pellet was homogenized for 30 sec and the homogenate was layered on a sucrose step gradient made of 10 ml of 80%, 7 ml of 60%, and 7 ml of 50% (w/v) sucrose in 0.02 M PB, pH 8.2. The gradient was centrifuged for 1 hr at 21,000 rpm in a Beckman SW 25.1 rotor. The inclusions, which layered on top of the 80% sucrose zone, were collected dropwise from the bottom of the tube. To remove the sucrose, the inclusions were diluted in 0.02 M PB, pH 8.2, and pelleted by centrifugation at 27,000 *g* for 15 min. The pellet was resuspended in 0.02 M Tris, pH 8.2, and inclusion yield was estimated spectrophotometrically after disruption in 2% sodium dodecyl sulfate (SDS). The inclusion preparations either were used immediately for immunization of rabbits or stored at −20 C by either freezing directly or by freeze-drying.

Clarification with *n*-butanol and chloroform-carbon tetrachloride. Because *n*-butanol resulted in virus preparations of higher purity, but chloroform-carbon tetrachloride was superior for preservation of inclusion proteins (E. Hiebert, *unpublished*), these solvent systems were combined for purification of virus and inclusions from the same batch of tissue. Infected tissue was homogenized with two parts (w/v) of 0.5 M PB, pH 7.5, containing 0.5–1.0% Na_2SO_3. The homogenate was filtered through cheesecloth and subjected to centrifugation at 11,700 *g* for 10 min. The supernatant was used for virus purification as described previously, with *n*-butanol for clarification. The pellet was resuspended in approximately two volumes of 0.5 M PB, pH 8.2, containing 0.5% Na_2SO_3, homogenized with one volume of chloroform-carbon tetrachloride (1:1,v/v) and centrifuged at 4,000 *g* for 5 min. The aqueous phase was centrifuged at 11,700 *g* for 15 min. The supernatant was collected for additional virus purification using PEG, equilibrium density-gradient centrifugation, and differential centrifugation. The pellet was resuspended in 0.05 M PB, pH 8.2, containing 0.1% 2-ME and treated with 5% Triton X-100. The inclusions then were purified by sucrose step-gradient centrifugation as described above.

Virus particle size determination. Crude leaf extracts from systemically infected cowpea plants and purified virus preparations were negatively stained in 2% potassium phosphotungstate (PTA), pH 6.5, containing 0.1% bovine serum albumin. The virus particles were observed in a Philips Model 200 electron microscope, and their sizes were estimated by comparing projected micrographs to micrographs of a diffraction grating (2,160 lines per millimeter).

Stability of virus in sap. Thermal inactivation point (TIP), longevity in vitro (LIV), and dilution end point (DEP) were determined for BlMC by using *Chenopodium amaranticolor* Coste and Reyn. as an assay plant. All test plants were maintained in a greenhouse for at least 3 wk for observations of symptoms.

Polyacrylamide gel electrophoresis of viral and inclusion proteins. The polyacrylamide gel electrophoresis studies were performed by the method of Weber and Osborn (32) as modified by Hiebert and McDonald (12).

Sedimentation coefficient determination. The sedimentation rates of fresh and stored purified BlCMV in either 0.02 M Tris buffer, pH 8.2 or 0.05 M borate buffer, pH 8.2, were estimated with a Beckman Model E analytical ultracentrifuge (20). After the rotor reached a speed of 27,690 rpm, photographs were taken at 4-min intervals through Schlieren optics. The rates were corrected to water viscosity at 20 C. The virus concentrations used varied from 0.5 to 1.0 mg/ml.

Serology. *Antiserum production for virus and cytoplasmic inclusions.* Antisera were obtained by injecting a New Zealand white rabbit with untreated virions and a second rabbit with protein from pyrrolidine-dissociated virus (27). The concentrations of purified BlCMV used in the immunization process varied from 1.0 to 2.0 mg of nucleoprotein per ml.

A series of four to five intramuscular injections was given to each rabbit with an interval of 10 to 15 days between the injections. Each injection consisted of 1.0 to 2.0 ml of virus or dissociated viral protein emulsified with an equal volume of either Freund's complete or incomplete adjuvant (Difco). Booster injections were given at intervals of about 2 mo. The immunized animals were bled every week, starting 10–15 days after the last injection of the initial series of four or five injections. The antisera were stored at −20 C by either freezing directly or after freeze-drying.

The BlCMV-induced cytoplasmic inclusions used for antiserum production were purified from *N. benthamiana*. Freshly purified cylindrical inclusions, which were unreactive with BlCMV antiserum, were used for the foot pad route of immunization (35). The rabbit received three injections, each containing 0.1 ml of purified inclusions (A_{280} = 1.0–2.0) in 0.02 M Tris, pH 8.2, emulsified with an equal volume of either Freund's complete or incomplete adjuvant.

Serological tests. Most double immunodiffusion tests were performed in agar medium containing 0.8% Noble agar (Difco); 0.5% SDS (Sigma) and 1.0% NaN_3 (Sigma) either in deionized water (25), or in 0.05 M Tris-HCl buffer, pH 7.2. Reactant wells were punched in the solidified agar medium with an adjustable gel-cutting device made by Grafar Corp., Detroit, MI 48238. Routinely, the wells (7 mm in diameter) were punched in a hexagonal arrangement consisting of a center well with six peripheral wells 4–5 mm away as measured from the nearest edges of the wells. Antigens used as reactants were prepared either in deionized water or in 1.5% SDS solution (25). The antigens and undiluted antisera were pipetted directly into the appropriate wells, and the plates were incubated in a moist chamber at 24 C for 24-48 hr. Prior to photography, the reactant wells were emptied by aspiration and then filled with Norit A charcoal (15%, w/v) (25).

Serological relationship between BlCMV and other viruses. Reciprocal double immunodiffusion tests with BlCMV and the following potyviruses were conducted: bean yellow mosaic virus (BYMV), bean common mosaic virus (BCMV-BV-1), bean common mosaic virus siratro isolate (BCMV-S), bidens mottle virus (BiMV), dasheen mosaic virus (DMV), lettuce mosaic virus

(LMV), pepper mottle virus (PeMV), potato virus Y (PVY), soybean mosaic virus (SoyMV), tobacco etch virus (TEV), turnip mosaic virus (TuMV), watermelon mosaic virus-1 (WMV-1), and watermelon mosaic virus-2 (WMV-2). The source of each antiserum was as follows: BYMV (14); BCMV-BV-1 (J. K. Uyemoto, Kansas State University, Manhattan); BCMV-S (19); DMV (1); BiMV, LMV, PeMV, PVY, SoyMV, TEV, TuMV, WMV-1, and WMV-2 (D. E. Purcifull). All antigens were tested in freshly prepared extracts or resuspended freeze-dried extracts from infected plants.

With BlCMV-antiserum, the serological relationship of BlCMV with commelina mosaic virus (CoMV) (21), a Moroccan isolate of commelina mosaic virus (CoMV) (21), a Moroccan isolate of cowpea aphid-borne mosaic (CAMV) (10), and pepper veinal mottle virus (PVMV) also were studied in double diffusion tests with SDS-treated antigens. In all serological tests, the reactants were arranged so that BlCMV always was placed in a well adjacent to the other virus-well. Sap extracts from appropriate healthy host tissues were included as controls in all serological tests and all antigens also were tested against normal serum.

The intragel cross-absorption technique (31) was also used to study the serological relationships of BlCMV with BCMV-S and CAMV.

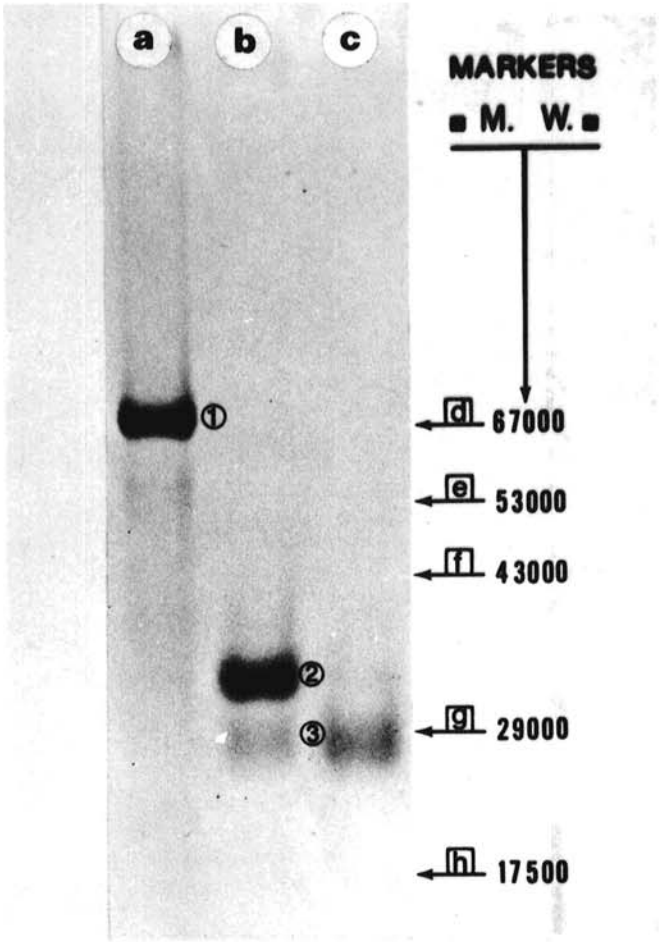

Fig. 1. Polyacrylamide gel electrophoresis analyses of (a) SDS-dissociated BlCMV cytoplasmic inclusions, (b) viral coat protein of freshly purified BlCMV, (c) and viral coat protein of BlCMV after 30 days of storage at 4 C. Migrations of marker proteins on companion lanes are represented by arrows: [d] bovine serum albumin (MW = 67,000); [e] glutamate dehydrogenase (MW = 53,000); [f] ovalbumin (MW = 43,000); [g] carbonic anhydrase (MW = 29,000); and [h] tobacco mosaic virus capsid protein (MW = 17,500). The components and their estimated molecular weights are: (1) BlCMV inclusion protein (MW = 70,000); (2) undegraded BlCMV coat protein (MW = 34,000); and (3) partially degraded viral coat proteins (two components, with MWs of about 29,000 and 27,000 were usually resolved).

Light and electron microscopy of virus-induced pinwheel inclusions. Epidermal leaf strips obtained from healthy and systemically infected cowpea were treated with Triton X-100, stained with Calcomine orange and "luxol" brilliant green as described by Christie and Edwardson (7), and examined by light microscopy.

Cylindrical inclusions were examined in situ in ultrathin sections with a Philips 200 electron microscope. Small pieces were taken from symptomatic areas of systemically infected cowpea leaves, fixed in formaldehyde-glutaraldehyde (15), postfixed in 2% osmium tetroxide, dehydrated, and embedded in Epon-araldite. Thin sections were stained according to Soloff (28). Purified inclusion preparations were mounted on carbon-coated Formvar film supported by copper grids and stained with either 1% ammonium molybdate or 2% uranyl acetate, before examination by electron microscopy.

Host range and screening cowpea cultivars for resistance. Test plants were inoculated with crude sap from Knuckle Purple Hull systemically infected with BlCMV. All inoculated plants including those that did not show any symptoms, were checked serologically for the presence of BlCMV.

Twenty cowpea cultivars also were inoculated with BlCMV, cowpea mosaic virus (CPMV), CAMV, and BCMV-S. Samples of crude sap from all inoculated cowpea plants also were tested serologically by double immunodiffusion.

RESULTS

Purification and properties of blackeye cowpea mosaic virus. Purified preparations of BlCMV were obtained from systemically infected leaves of either *V. unguiculata* 'Knuckle Purple Hull' or *N. benthamiana* using any of the purification procedures. The best yield with the highest degree of purity was obtained by using infected cowpea leaves as a source of virus and *n*-butanol for clarification. The first trifoliolate cowpea leaves collected 15–18 days after inoculation gave the highest yield of virus (8–10 mg/100 g of infected tissue).

The UV absorption spectrum had a maximum at 260–262 nm, and a minimum at 244–245 nm. The ratio of A_{260}/A_{280} was 1.2 after correction for light scattering. The virus solutions showed strong stream birefringence and electron micrographs indicated that 73% of the 190 virus particles examined were between 700 and 800 nm, with a modal length of 753 nm. The rods observed in the purified preparations indicated a low percentage of virus fragments. Particle measurements of several leaf-dip preparations negatively stained with PTA and of grids prepared for serologically specific electron microscopy with infected cowpea leaf tissue extracts gave modal lengths of 758 and 780 nm, respectively, with 90% of the particle lengths ranging from 700–800 nm.

Sedimentation coefficients determined for the virus at 20 C either in 0.02 M Tris buffer, pH 8.2, or in 0.05 M borate buffer, pH 8.2, indicated that BlCMV sedimented as a single species with the s_{20} values of 157–159S. On the other hand, the schlieren patterns revealed a difference in S values between BlCMV in fresh preparations and BlCMV in purified preparations stored at 4 C for more than 30 days. Both virus preparations showed a single sedimenting peak but the s_{20} values for BlCMV in fresh preparations and at concentrations varying from 0.5 to 1.0 mg/ml ranged from 157 to 159S while the s_{20} values for the virus in the stored preparations and at the same concentrations ranged from 140 to 142S.

Proteolytic degradation of capsid protein in stored, purified preparations of BlCMV was observed by polyacrylamide gel electrophoresis (PAGE). The PAGE analysis of a freshly purified preparation of BlCMV revealed a main protein component with an estimated molecular weight (MW) of 34,000 and two smaller ones with MWs of 29,000 and 27,000 (Fig. 1). Stored BlCMV preparations contained only the faster-moving protein components with MWs of 29,000 and 27,000 (Fig. 1), presumably derived from the 34,000 component.

In cowpea leaf extracts, BlCMV had a thermal inactivation point of 65 C, in vitro longevity of 48 hr, and a dilution end point of 10^{-4}.

Purified inclusion preparations. With both methods for inclusion purification, purified cytoplasmic inclusions induced by BlCMV and virus particles were obtained from the same batches of systemically infected leaf tissue of *V. unguiculata* or *N. benthamiana*. Electron microscopy of purified BlCMV-inclusions negatively stained with molybdate showed tubular inclusions with only trace amounts of host components (Fig. 2B). A few virus particles were observed in the purified preparations of inclusions, but the latter did not react with BlCMV antiserum in immunodiffusion tests (Fig. 3). The most highly purified preparations of inclusions were obtained from *N. benthamiana*, but yields of 5–20 A_{280} units usually were obtained from 100 g fresh weight of either *N. benthamiana* or *V. unguiculata* tissues. The UV absorption spectrum obtained for SDS disassociated BlCMV inclusions was typical of proteins, with a maximum at 277 nm and a minimum at 246–248 nm. Polyacrylamide gel electrophoresis of SDS-disrupted inclusion proteins revealed a single subunit component estimated to have a MW of 70,000 (Fig. 1).

Serology. The antisera obtained against untreated BlCMV and pyrrolidine-dissociated BlCMV were both reactive with SDS- or pyrrolidine-dissociated virus in purified preparations or in plant sap. The specificity of BlCMV antiserum in an SDS-double radial diffusion test is shown in Fig. 3. The titers of antisera varied depending on the bleeding date and on the rabbits, but 32 was the highest titer estimated by SDS-gel double diffusion tests.

Antiserum specific for cytoplasmic inclusions induced by BlCMV was obtained from a rabbit injected with preparations of inclusions purified from infected tissue of *N. benthamiana*. The inclusion antiserum reacted specifically with purified preparations of BlCMV inclusions and crude sap of BlCMV-infected cowpea, but not with either purified preparations of BlCMV or crude sap of noninoculated plants (Fig. 3). These findings are similar to those obtained with cylindrical inclusion proteins of other potyviruses (25).

Reciprocal double immunodiffusion tests with SDS-treated antigens showed that BlCMV is serologically related to, but distinct from, the following potyviruses: BCMV-BV-1, BCMV-S, BYMV, DMV, LMV, PVY, SoyMV, TEV, and WMV-2. No reactions were detected, however, with several other potyviruses, including BiMV, PeMV, TuMV, WMV-1, CoMV, and PVMV. Antiserum for BlCMV also reacted specifically with CAMV, forming a distinct spur which extended past the heterologous reaction (Fig. 4A).

The serological distinctions observed between BlCMV and either CAMV (Fig. 4A) or BCMV-S (Fig. 4C) by spur formation also were demonstrated by the intragel cross absorption technique (Fig. 4B and D). The heterologous antigens, which were placed in the center well prior to the antiserum, cross-reacted with and fully

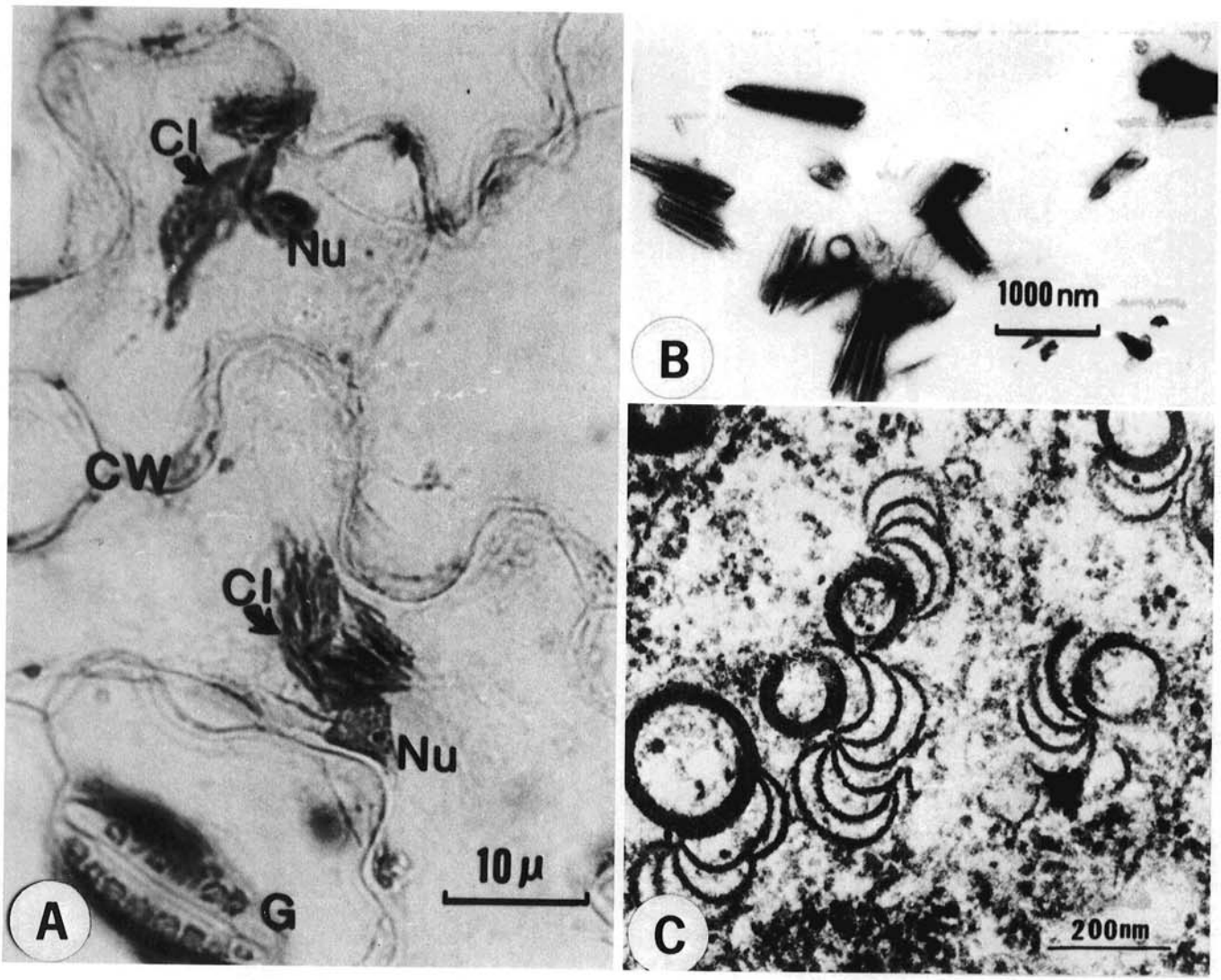

Fig. 2. Micrographs of BlCMV-induced cytoplasmic inclusions. **A,** Photomicrograph of cytoplasmic inclusions in an epidermal strip from a BlCMV-infected cowpea leaf, stained with a combination of Calcomine orange and Luxol brilliant green. Cytoplasmic inclusions (CI), plant cell wall (CW), guard cells (G), and nucleus (Nu); **B,** Electron micrograph of a purified preparation of BlCMV cytoplasmic inclusions stained with molybdate; and **C,** Electron micrograph of ultrathin section of cowpea leaf cell infected with BlCMV showing cross sections of pinwheel inclusions.

precipitated the cross-reacting antibodies at the region of optimal proportions close to the center well.

The precipitin line formed by the reaction of BlCMV spurred over the lines formed with purified BlCMV which had been stored for more than 1 mo at 4 C.

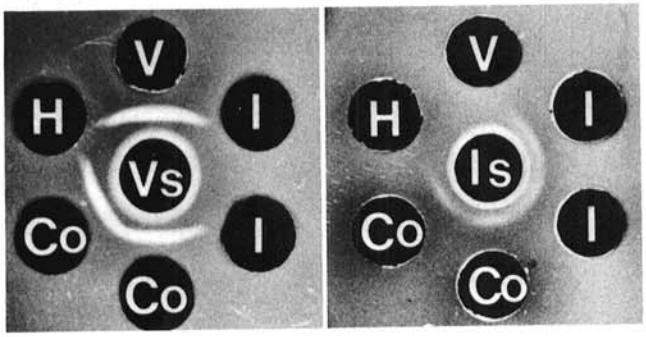

Fig. 3. Serological tests to demonstrate specificity of BlCMV antiserum (Vs), and antiserum obtained against BlCMV-induced cytoplasmic inclusions (Is). The peripheral wells were filled with the following antigenic solutions prepared in 1.5% SDS: (V) purified BlCMV, (I) purified preparations of BlCMV inclusions, (Co) extracts of BlCMV-infected cowpea, and (H) crude extract of healthy cowpea. Immunodiffusion medium consists of 0.8% Noble agar, 1.0% NaN_3, and 0.5% SDS.

Light and electron microscopy. Light microscopic observations of epidermal leaf strip preparations from cowpea plants systemically infected with BlCMV revealed tubular cytoplasmic inclusions (Fig. 2A) and cylindrical inclusions (Fig. 2C) similar to those reported for BlCMV in other hosts (7,8,9,33).

Host range and resistant cowpea varieties. Blackeye cowpea mosaic virus was readily transmitted mechanically from cowpea (Knuckle Purple Hull) to the following plants in which it was detected serologically (symptoms listed in parentheses): *Chenopodium amaranticolor* (small chlorotic lesions); *Crotalaria spectabilis* (mosaic); *Glycine max* (L.) Merr. (mild mottle and chlorotic spots); *Macroptilium atropurpureum* (DC.) Urb. (mosaic); *Nicotiana benthamiana* Domin (mottle); *Ocimum basilicum* (local lesions); *Phaseolus vulgaris* 'Black Turtle-2' (epinasty, necrosis, yellowing) and 'Bountiful' (chlorotic spots on inoculated leaves); *Vigna unguiculata* 'Black Local' (mosaic), 'Early Ramshorn' (mottle), 'Knuckle Purple Hull' (mosaic), and 12 Brazilian cowpea cultivars in which the reactions varied from symptomless to mosaic (Table 1).

Based on failure to induce symptoms and on negative serological results, BlCMV did not infect *Arachis hypogaea* L. 'Florunner', *Capsicum annuum* L. 'Early Calwonder'; *Cucumis sativus* L.; *Cucurbita pepo* L. 'Small Sugar'; *Lupinus angustifolius* L. 'Bitter

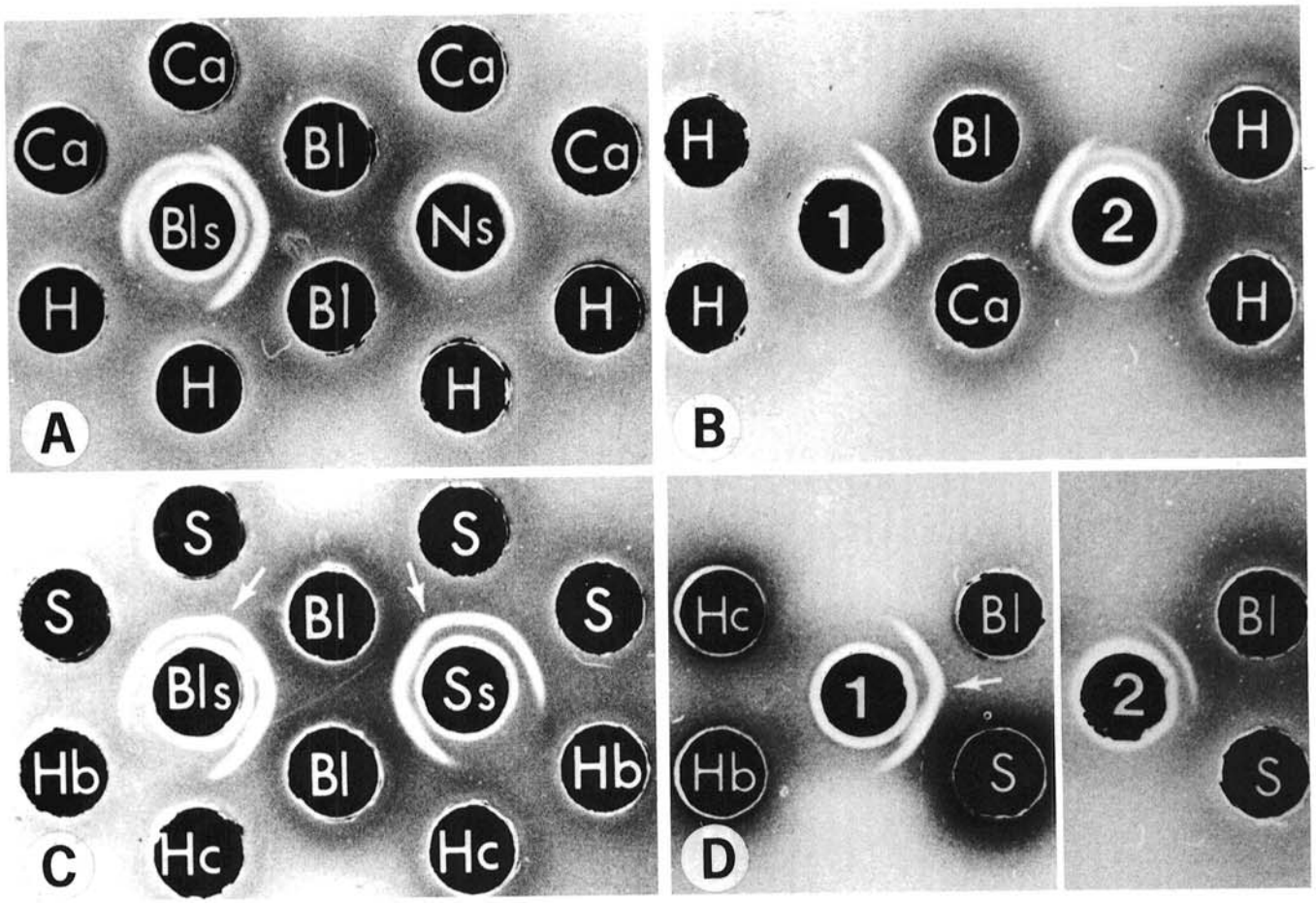

Fig. 4. Immunodiffusion tests with BlCMV, Moroccan isolate of CAMV and siratro strain of bean common mosaic virus (BCMV-S) in agar medium containing 0.8% Noble agar, 1.0% NaN_3, and 0.5% SDS prepared in 0.05 M Tris-HCl buffer, pH 7.2. **A,** Serological tests with BlCMV and CAMV. The center wells were charged with: BlCMV antiserum (Bls), and normal serum (Ns). The peripheral wells were filled with SDS-treated extracts from: (Bl) BlCMV-infected cowpea, (Ca) CAMV-infected cowpea, and (H) healthy cowpea. **B,** Intragel cross-absorption test with BlCMV and CAMV. The center wells were charged with: (1) BlCMV antiserum, (2) purified CAMV and 20 hr later BlCMV antiserum. The peripheral wells were filled with SDS-treated extracts from: (Bl) BlCMV-infected cowpea, (Ca) CAMV-infected cowpea, and (H) healthy cowpea. **C,** Serological tests with BlCMV and BCMV-S. The center wells were charged with: (Bls) BlCMV antiserum, (Ss) BCMV-S antiserum. The peripheral wells were filled with SDS-treated extracts from: (Bl) BlCMV-infected cowpea, (S) BCMV-S infected bean, (Hb) healthy bean, and (Hc) healthy cowpea. The arrows point to locations of faint spurs which were detectable by direct observation of the plates. **D,** Intragel cross-absorption test with BlCMV and BCMV-S. The center wells were filled with: (1) BlCMV antiserum and (2) purified BCMV-S and 20 hr later BlCMV antiserum. The peripheral wells were charged with SDS-treated extracts from: (Bl) BlCMV-infected cowpea, (S) BCMV-S infected bean, (Hb) healthy bean, and (Hc) healthy cowpea.

TABLE 1. Symptoms and results of serological assays on cultivars of cowpea, *Vigna unguiculata,* mechanically inoculated with blackeye cowpea mosaic (BlCMV), bean common mosaic—siratro isolate (BCMV-S), cowpea aphid-borne mosaic (CAMV), and cowpea mosaic (CPMV) viruses

Cowpea cultivars	Symptoms[a]				Serology[b]			
	BlCMV	BCMV-S	CAMV	CPMV	BlCMV	BCMV-S	CAMV	CPMV
Black Local	Mt	−	M	−	+	−	+	−
Bola de Ouro	−	−	Mt	Mt	−	−	+	+
CE-73	−	Mt	Mt	Mt	+	+	+	+
CE-74	Mt	−	Mt	−	+	−	+	−
CE-175	M	−	Mt	Mt	+	−	+	+
CE-89	M	−	Mt	−	+	−	+	−
CE-3-53	Mt	−	M	−	+	−	+	−
Cream 40	Mt	−	M,Ld	M, Ld, De	+	−	+	+
Early Ramshorn	Mt	M	M	Ne,De	+	+	+	+
Crowder Pea	−	−	Mt	M,Ld	−	−	+	+
Ipeane VIII	Mt	−	Mt	−	+	−	+	−
Jaguaribe	M	−	Mt	−	+	−	+	−
Knuckle Purple Hull	M	−	M,Ld	M,Ld,De	+	−	+	+
Pitiuba	Mt	−	M	−	+	−	+	−
Potomac	M	−	Mt	−	+	−	+	−
Serido	−	−	Mt	Mt	−	−	+	+
Snapper Long Pod	−	−	M,Ld	···	−	−	+	···
Sete Semanas	−	−	Mt	−	−	−	+	−
V-4 Alagoas	M	−	Mt	Chl	+	−	+	+
V-5 Parayba	M	−	M	Mt	+	−	+	+

[a]Abbreviations: Chl = systemic chlorosis, De = plant death, Ld = leaf deformation, M = mosaic, Mt = mottle, Ne = systemic necrosis, and − = no symptoms.
[b]Symbols: + = positive serological reaction in sap in double diffusion tests; − = no serological reaction in double diffusion tests; and ··· = not tested.

Blue'; *Lupinus luteus* L. 'Sweet Yellow'; *Phaseolus vulgaris* 'Black Turtle-1', 'Green Northern 1140', 'Improved Tendergreen', 'Lake Shasta', 'Michelite 62', 'Pink Rosa', 'Pink Viva', 'Puregold Wax', 'Red Mexican U-34', 'Red Mexican U-35', 'Top Crop', and 'VC 1822'; *Pisum sativum* L. 'Alaska', 'Bonneville', and 'Ranger'; and *Vicia faba* L.

The reactions of cowpea cultivars to mechanical inoculations of BlCMV, BCMV-S, CAMV, and cowpea mosaic virus (isometric) are indicated in Table 1. All inoculated plants were assayed serologically for the presence of the viruses (Table 1).

DISCUSSION

Blackeye cowpea mosaic virus and its cytoplasmic inclusions were purified from systemically infected cowpea or *N. benthamiana* leaf tissues by employing the procedures described herein. The high degree of purity of the BlCMV preparations, which was indicated by spectrophotometry, analytical centrifugation, PAGE analysis, serological studies, and electron microscopic observations, confirmed the efficiency of the purification procedures.

Polyacrylamide gel electrophoresis of SDS-disassociated cytoplasmic inclusions and viral coat proteins clearly indicated that the viral coat protein subunit was smaller than the inclusion subunit. Since only traces of the faster-moving proteins were observed with freshly purified BlCMV, and greater amounts of these proteinaceous components were revealed by PAGE analysis of stored purified BlCMV preparations, it is assumed that the smaller components are due to the degradation of the coat protein subunit during purification and storage. Hiebert and McDonald (13) observed possible enzymatic degradation of TuMV capsid protein that occurred during storage of purified virus preparations. The lower sedimentation coefficient estimated for stored purified BlCMV preparations is additional evidence that viral coat protein is degraded during storage at 4 C. Furthermore, results of immunodiffusion analyses indicated that purified BlCMV stored at 4 C for more than 30 days lacked some antigenic determinants which were present in freshly purified BlCMV. The significance of such antigenic alterations for serological identification and characterization of potyviruses has been pointed out previously (13,25).

Unilateral serological relationships observed between BlCMV and SoyMV and between BlCMV and BYMV showed the necessity of reciprocal tests for demonstrating the absence of serological relationship between the two viruses. Reciprocal tests also were useful for demonstrating distinctions between two closely related viruses. It was more difficult to observe a spur between BlCMV and WMV-2 when both viruses were tested against BlCMV antiserum than in the reciprocal test. Similar results were observed with BCMV isolates and BlCMV, which may explain the identical reaction reported by Uyemoto et al (29).

Although related, BlCMV and BYMV are serologically distinct. This supports the contention of Edwardson et al (9), Zettler and Evans (34), and Pio-Ribeiro et al (23) that BlCMV and BYMV should be considered only distantly related viruses.

Serological relationships between different potyviruses often have been observed (4,26,27,30), and cross-absorption tests have been useful for studying the relationship between potyviruses in gel diffusion tests (14,26,33). The intragel cross-absorption test (31) effectively demonstrated distinctions between two viruses that are serologically closely related (BlCMV and BCMV-S or CAMV).

The three serologically related but distinct legume viruses, BlCMV, BCMV-S, and CAMV, also can be differentiated by some biological properties. The CAMV isolate was well adapted to cowpea, infecting and causing symptoms in all 20 inoculated cowpea cultivars. On the other hand, five cowpea varieties showed immunity to BlCMV, and only two were infected with BCMV-S, which caused very mild symptoms (Table 1). The different symptoms induced by CAMV and BlCMV clearly indicate that some cultivars (Table 1) can be used to distinguish these two potyviruses. It was observed, however, that some of the symptoms induced by the viruses varied with temperature and light conditions, and age at which the plants were inoculated, but no variation was observed with respect to the immunity of any cowpea cultivar. The cowpea cultivars that showed immunity to BlCMV (Table 1) should be included in a cowpea breeding program or in a program designed to control this virus in the southeastern USA.

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