Purification, Properties, and Serology of Carnation Yellow Fleck Virus

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Contribution No. 220-E, 1975 Series, from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

The authors wish to thank: G. Loebenstein for help and constructive criticism throughout the preparation of this work, Orit Warman for technical assistance, and J. Cohen for help with the electron microscopy.

Accepted for publication 15 December 1975.

ABSTRACT

BAR-JOSEPH, M., and M. SMOOKLER. 1976. Purification, properties, and serology of carnation yellow fleck virus. Phytopathology 66: 835-838

Carnation yellow fleck virus (CYFV) was partially purified from spray carnations, *Dianthus caryophyllus*, by clarification with bentonite, concentration with 4% polyethylene glycol, and further purification by rate zonal and isopyonic gradient centrifugation. These methods enabled almost complete removal of carnation mottle virus (CarMV) particles present in the plant material. The purified particles had an unusual ultraviolet absorption spectrum ($A_{260/280} = 1.53$), contained about 5% RNA (estimated from a buoyant density $p = 1.325$ in CsCl), with an extinction coefficient of $E^{0.1\%}_{260} = 2.27$. The molecular weight of the coat protein subunit was about 23,500. In the serological ring precipitin test, CYFV antiserum had a titer of 1/256 and 1/8 against dissociated virus (CYFV-D) in the gel diffusion tests. The antisera did not react with extracts from healthy plants nor with those from plants infected with CarMV, carnation etched ring, and carnation vein mottle viruses.

Carnation yellow fleck virus (CYFV), an elongated virus that can infect carnations, has recently been described (15). The disease causes yellow mottling, streaking, flecking, necrosis, and purple discoloration of carnation leaves, and is transmitted semipersistently by *Myzus persicae*. Carnation yellow fleck virus was transferred mechanically, but with extreme difficulty. Morphologically, CYFV closely resembled beet yellows virus (BYV) which also has a modal length of 1,250 nm, but did not react with BYV antiserum. A similar disease has been reported from Australia (16), Japan (7), and New Zealand (K. S. Milne, *personal communication*).

This communication reports on a purification procedure that separates CYFV from carnation mottle virus (CarMV), further properties of the particle and preparation of an antiserum.

MATERIALS AND METHODS

Since plant material infected with CYFV alone and grown in a screened greenhouse was limited, infected material was collected from *Dianthus caryophyllus* L. 'Cerise Royallet' plants, grown commercially at Kohav. These plants were usually infected also with CarMV. Only those parts of the leaf with strong symptoms of yellow mottling and flecking were taken for purification.

Purification.—Twenty-five-gram batches of frozen leaves were homogenized in 200 ml 0.05M sodium phosphate buffer (PB), pH 7.3, with the aid of a "VirTis" homogenizer in the cold. The homogenate was squeezed through cheesecloth and partially clarified in a Sorvall GSA rotor first at 4,000 *g* for 5 minutes then at 8,000 *g* for 10 minutes. The supernatants were collected gently and mixed with 7 ml of a 45 mg/ml bentonite solution prepared according to Lister and Hadidi (8). After centrifugation at 5,800 *g* for 10 minutes, 4 *g* polyethylene glycol 6000 (PEG) and 4 ml of 20% NaCl were added to each 96 ml of supernatant. The PEG was dissolved by stirring and the solution was kept in the cold for 30 minutes, before being centrifuged for 10 minutes at 16,300 *g*. The pellets were dissolved by stirring in one-seventh of the original volume in water. After two low-speed centrifugations at 5,900 and 9,800 *g* each for 10 minutes (Sorvall SS-34 rotor), 1 g PEG and 1 ml 20% NaCl were dissolved in 24 ml supernatant, and the precipitate was collected by centrifuging at 12,000 *g* for 10 minutes. The pellets were soaked in 3 ml water overnight, followed by 30 minutes of stirring, all steps being carried out in the cold. This suspension, after two further clarifications at 5,900 and 9,800 *g* for 10 minutes each, was designated as the partially purified preparation.

Density-gradient centrifugation.—Rate zonal density gradients (4) were made by layering 5, 7, 7, and 7 ml of solution containing 10, 20, 30, and 40% (w/v) sucrose, respectively, in 0.025 M PB pH 7.3, and keeping the tubes at 4 C overnight. Two milliliters of partially purified virus preparation was layered on top of each of the gradients, which were then centrifuged for 2.5 hours at 22,500 rpm in

a Spinco SW 25.1 rotor. The density-gradient tubes were then analyzed using an ISCO UA-5 ultraviolet analyzer and the virus-containing fractions were collected using an ISCO 640 fraction collector. The virus was precipitated from the sucrose solution by adding half a volume of a 30% PEG in 0.6 M NaCl solution. The mixture was kept overnight in the cold, centrifuged for 10 minutes at 7,700 *g*, and the pellets were resuspended in water.

Equilibrium density-gradient centrifugations were made in cesium chloride (CsCl) in a Spinco SW 39 rotor. A mixture containing 0.1 ml partially purified CYFV (1mg/1ml), 1.1ml CsCl ($p = 1.79$) and 2.6ml 0.01M PB, pH 7.3, was layered on top of 1.0 ml CsCl ($p = 1.79$) in 13 × 51-mm tubes. For comparison, tobacco mosaic virus (TMV) was added to one of the tubes by replacing 0.03 ml PB with the same volume of TMV (1 mg/1 ml). The tubes were centrifuged for 20 hours at 35,000 rpm at 4 C. The density gradient tube was then analyzed as described above. The refractive indices of various fractions were measured using a Zeiss refractometer and these values were converted to density by the formula of Vinograd and Hearst (17).

Disc electrophoresis.—A 5% polyacrylamide formulation containing 0.1% sodium dodecyl sulfate (SDS) was used according to Maizel (9). Dissociation of virus preparation and marker proteins, electrophoresis, and staining were as described previously (3).

Serology.—Carnation yellow fleck virus antisera were prepared by injecting rabbits with partially purified preparations. Each rabbit was injected intramuscularly five times at weekly intervals with about 2 mg of virus emulsified in Freund's complete adjuvant. Subsequently, five injections with 1 mg virus were given at 3-day intervals.

Antiserum was also prepared against CYFV dissociated protein (CYFV-D). Purified preparation was dissociated using pyrrolidine (14). For intravenous injections pyrrolidine was removed by dialysis (14). For intramuscular injections CYFV-D was recovered from the pyrrolidine solution by two cycles of precipitation with 10% PEG and 0.2 M NaCl and centrifugation for 15 minutes at 12,000 *g*. The final precipitate was resuspended in 0.01 M sodium phosphate buffered saline (PBS), pH 7.3, and emulsified with adjuvant. The injection schedule and amount of antigen were similar to those described for whole virus. The rabbits were bled 4 weeks after the first injection. For absorption with host proteins, 200 g CYFV-free carnation leaves were homogenized in 600 ml PB. After clarification, the supernatant was brought to 8% PEG and 0.2 M NaCl. After centrifugation for 10 minutes at 5,800 *g*, the resuspended pellets were dissociated in 2.5% pyrrolidine. Pyrrolidine was removed by two cycles of precipitation with 10% PEG and 0.2 M NaCl and centrifugation for 15 minutes at 12,000 *g*. The final pellet was dissolved in 4 ml 0.01 M PBS, pH 7.3. Ten ml antiserum was incubated for 1 hour at 37 C with 2 ml host proteins. The mixture was then left overnight at 4 C and the precipitate was removed by low-speed centrifugation. The antisera were diluted 1:1 with glycerol and stored at −10 C.

For ring interface precipitin tests (10), antiserum was diluted serially with PBS containing 10% glycerol. Antiserum was overlain with different dilutions of partially purified virus and the tubes were incubated for 30 minutes in a water bath at 37 C.

The medium for immunodiffusion tests consisted of 1.5% Noble agar in 0.05 M Tris [tris(hydroxymethyl)-aminomethane]-HCl buffered saline, pH 7.2, and 0.02% sodium azide. Tests were done in 5.5-cm diameter petri dishes covered with formvar.

Juice was extracted from symptom-expressing leaves with a hand press and a pyrrolidine solution was added to a final concentration of 2.5% to dissociate the virus. Partially purified virus was treated similarly.

RESULTS AND DISCUSSION

Purification.—A single band, about 25 mm below the meniscus (peak fractions 9-11, Fig. 1), was obtained after rate zonal centrifugation. The peak fraction contained high concentrations of CYFV particles, apparently free of host material (Fig. 2). A few CarMV particles were observed in some of the fractions. CarMV particle counts, in comparison with those of CYFV and local lesion assays on *Chenopodium amaranticolor,* related to standard infectivity curves, indicated that CarMV concentration, even in fraction 12, was only 0.06% of CYFV. The average yield from more than 20 purifications obtained during February-March 1975, was 2.8 mg/25 g leaves, ranging from 1.3 - 5.3 mg/25 g leaves.

Physical properties.—Analytical ultracentrifugation indicated a sedimentation coefficient S_{20} (0.025 PB, pH 7.3) of 80 at a concentration of 2.8 mg/ml.

Carnation yellow fleck virus formed a single band in CsCl gradients at $p = 1.325$ g/ml, cobanding with tobacco mosaic virus (TMV). However, by scanning fractions by electron microscopy it was observed that TMV was more

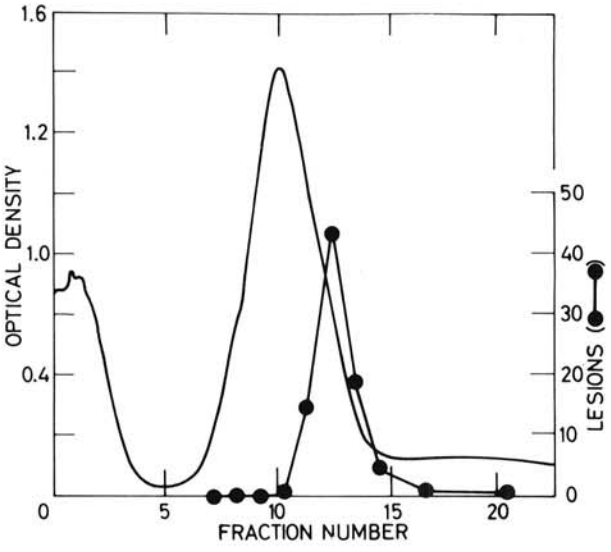

Fig. 1. Ultraviolet absorbance profile of rate zonal density-gradient centrifugation, of a partially purified carnation yellow fleck virus preparation from 25 g of carnation leaves. The gradient column was fractionated into 1.2-ml fractions. Carnation mottle virus infectivity in different fractions was examined on six half-leaves of *Chenopodium amaranticolor* after diluting 0.1 ml of the examined fraction in 10 ml of 0.025 M sodium phosphate buffer, pH 7.3.

concentrated in the heavier fractions than CYFV, indicating a somewhat lower density. An RNA content of about 5% may therefore be estimated (13). Carnation yellow fleck virus, unlike BYV (1), is stable in CsCl.

The ultraviolet absorption spectrum of a rate zonal purified preparation, after removing the sucrose by two cycles of precipitation with 10% PEG and 0.2 M NaCl, had an $A_{260/280}$ ratio = 1.53 and $A_{max/min}$ = 1.29 (Fig. 3). The $A_{260/280}$ is higher than expected for a nucleic acid content of about 5% (11), although even higher ratios of 1.72 and 1.73 have been reported for apple chlorotic leafspot virus (CLSV) and sugar beet yellows virus (BYV), respectively (1, 2). It is possible that the lower $A_{260/280}$ ratio of CYFV compared with CLSV and SBYV is due to a higher aromatic acid content in the CYFV protein.

The extinction coefficient of CYFV at 260 nm, as determined from three spectrophotometric and dry weight measurements (18), was 2.27 ± 0.1/mg.

Electrophoresis of CYFV coat protein in SDS - polyacrylamide gels revealed one band. With bovine serum albumin, ovalbumin, carbonic anhydrase, potato virus X, and TMV coat proteins as markers, the molecular weight of CYFV coat protein was estimated to be 23,500 ± 500. This is close to the molecular weight of the coat proteins of citrus tristeza virus (3), CLSV (2, 5), and BYV (1).

Serology.—Carnation yellow fleck virus antiserum collected 4 weeks after the first injection had an antibody dilution titer of 1/256 in ring interface precipitin tests, with no change for 3 months. In gel diffusion tests this antiserum had a titer of 1/8, whereas CYFV-D antiserum had a titer of 1/16 after 3 months. No "spur" formation was observed when CYFV and CYFV-D antisera were tested against CYFV-D antigen, perhaps due to partial degradation of the virus during immunization, or to cross reactivity of the antibodies.

Relatively poor immunogenicity seems to be a common feature with other long, flexuous viruses; i.e., 1/128-1/256 for CLSV (5, 6) and 1/128-1/512 for BYV (12; and Bancroft and Bar-Joseph, *unpublished*). CYFV-D antisera (after absorption with host proteins) did not react with extracts from healthy plants nor with those from plants infected with CarMV, carnation etched ring, or carnation vein mottle viruses.

A good correlation was obtained when serological indexing of infected plants was compared with indexing by electron microscopy. Out of 102 plants showing symptoms, and which indexed positive by electron microscopy, 93 (92%) gave a positive reaction in gel diffusion tests. The failures (8%) in serological detection were probably due to low virus titer in the plant source.

No serological reaction was obtained when symptomless parts of infected plants were assayed serologically, even after a 10- to 100-fold concentration of the plant extracts. Apparently, particle concentration in symptomless parts is low, and indeed very few particles, if any, can be detected by electron microscopy. Therefore, at present, antisera are useful mainly for establishing CYFV identity of plants evincing necrotic symptoms.

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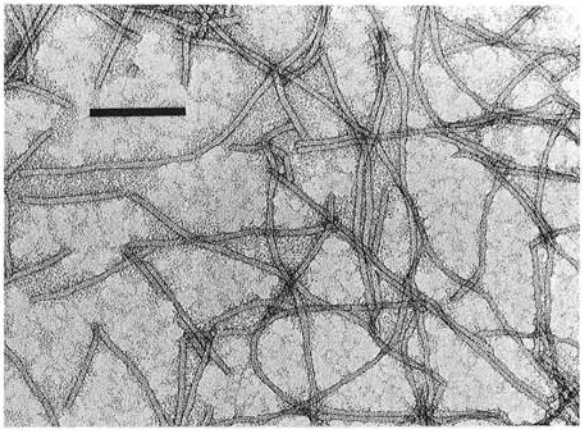

Fig. 2. Electron micrograph of a purified preparation of carnation yellow fleck virus negatively stained with uranyl acetate. Scale bar = 200 nm.

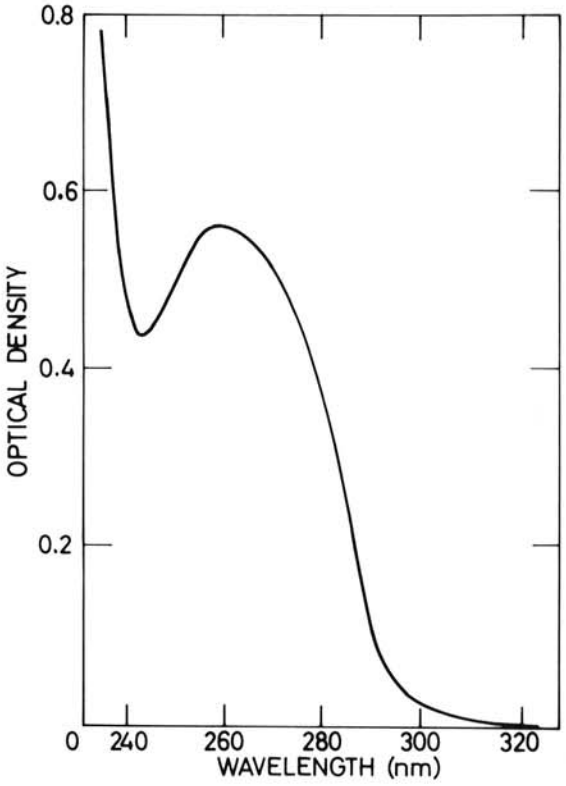

Fig. 3. Ultraviolet absorption spectrum of purified preparation of carnation yellow fleck virus in 0.1% sodium dodecyl sulfate.

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