

Purification of a Mild Mottle Strain of Peanut Mottle Virus

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ABSTRACT

Infectivity assays and electron microscopic examinations of various steps of purification indicated that particles of a mild mottle strain of peanut mottle virus severely aggregated when concentrated by differential ultracentrifugation and polyethylene glycol precipitation. Aggregation was partially overcome, however, when polyethylene glycol precipitated virus particles were resuspended in phosphate buffer containing 0.001 M Cleland's reagent (dithiothreitol). This procedure allowed the formation of a single opalescent zone in

density-gradient columns. The absorption spectrum of the purified virus was typical for nucleoproteins, with minimum and maximum absorbance at 246 and 260 nm, respectively. The most frequent length in the modal distribution of the particles was about 725 nm. Antiserum from an immunized rabbit had a specific titer of 1:256 and 1:128 with ring interface and microprecipitin tests, respectively.

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Peanut mottle virus (PMV) causes a disease of peanut (*Arachis hypogaea* L.), characterized by subtle mottling of developing leaves, which is widely prevalent in the Southeastern USA. Similar peanut virus diseases have been noted in Australia (2), Bulgaria (22), Japan (17), and Venezuela (14). Although these virus isolates produce similar symptomatology on peanuts and have similar in vitro physical properties, they can be differentiated by their reactions on various hosts. Furthermore, reported virus particle lengths for the different isolates range from 704 to 812 nm. In Georgia, several PMV isolates have been found (21) and the determination of the relationships among these peanut virus isolates is dependent on a satisfactory purification procedure.

As expected with a flexuous rod-type virus (5), PMV is difficult to purify and most procedures cause major or total losses of infectivity (20). Recently, Sun & Hebert (24) developed a scheme for purifying a severe mosaic strain of PMV (PMV-S). It involves chloroform-butanol extraction, polyethylene glycol precipitation, ultracentrifugation, and the use of urea in the suspending buffer. Butanol (washed or

unwashed with sodium bisulfite) and ultracentrifugation caused almost total loss of infectivity of our mild mottle PMV isolate (PMV-M). In direct comparison studies, PMV-M particles appeared to aggregate more readily than PMV-S, and perhaps more importantly, the concentration of PMV-M was several times less than PMV-S in peanut and in the best culture host, *Pisum sativum*. Since PMV-M is by far the most prevalent PMV isolate in Georgia (19, 21) and North Carolina (7) fields, it is important to find a purification procedure for this isolate.

MATERIALS AND METHODS.—*Virus source.*—A stock culture of a mild mottle isolate (PMV-M2) obtained from peanut fields in Georgia was maintained in Argentine peanut and *Pisum sativum* L. 'Little Marvel' in the greenhouse. Inoculum was made by grinding infected tissue in 0.01M potassium phosphate buffer, pH 8.0, containing 0.01M sodium diethyldithiocarbamate (DIECA) and 0.01 M sodium bisulfite (NaHSO_3), and 1% Celite.

Infectivity assay.—Infectivity levels of each step of purification were determined by local lesion assays on *Phaseolus vulgaris* L. 'Topcrop'. Treatment

comparisons were made on a half-leaf basis with an incomplete block design with treatment replications ranging from 6 to 12.

Purification.—The virus was purified by polyethylene glycol precipitation (15) and density-gradient centrifugation. Systemically infected leaves of Little Marvel pea were grown in the greenhouse (24 to 32 C) and harvested 9 to 12 days after inoculation and homogenized in a Waring Blender with 2 ml/g of the cold freshly prepared phosphate buffer described above. The extract was expressed through cheesecloth and shaken with 8 to 10% cold chloroform for 3 to 4 min, and the emulsion was broken by low speed centrifugation (12,000 g for 10 min). Polyethylene glycol 6000 (PEG) and potassium chloride (KCl) were added with stirring to the clarified extract to give a concentration of 4% and 0.2 M, respectively. The mixture was allowed to stand for an hour before low-speed centrifugation. The supernatant was discarded and the pellets were resuspended overnight in 0.05 M phosphate buffer, pH 8.0, containing 0.001 M Cleland's reagent (dithiothreitol). Further purification was obtained by a second PEG-KCl precipitation followed by rate zonal density-gradient centrifugation. The sucrose gradient was prepared by layering, respectively, 4, 7, 7, and 7 ml of 100, 200, 300 and 400 mg sucrose/ml of the suspending buffer. The column was stored at least 16 hr before use. Two ml of virus preparation were layered on each tube and centrifuged at 60,000 g for 4 hr in a Spinco SW 25.1 rotor. The gradients were then analyzed and fractionated with an ISCO density-gradient fractionator attached to an ultraviolet analyzer (254 nm). Virus zones were collected and used to determine infectivity, particle characteristics, and to produce antiserum.

Electron microscopy.—Purified virus samples were either mixed with 0.001% bovine serum albumin, sprayed on Formvar coated grids, and shadowed with 60:40 gold-palladium metal or negatively stained with 2% potassium phosphotungstate (KPT) at pH 7.2. The KPT was mixed with the virus preparation and a droplet was placed on the grid for a few seconds before excess liquid was drawn off with strips of filter paper. The leaf dip method of Brandes (4) was also employed. All examinations were made with either an RCA 2E or Philips EM-200 electron microscope. Measurements of virus particles were made on negatively stained preparations on enlarged prints with a magnification of ca. 50,000.

Serology.—Antiserum to PMV-M2 was produced in a rabbit injected with virus from the density gradient zone only. Alternate intravenous and intramuscular injections were given at weekly intervals for 6 weeks. The virus was emulsified with Freund's complete adjuvant before the intramuscular injection. One week after the last injection, the rabbit was bled, and the serum was stored at -29 C. Preparation of antiserum against normal plant protein was accomplished by extracting proteins from healthy Little Marvel pea with the PMV extraction buffer, precipitating twice with PEG-KCl, and injecting the suspension of the second precipitate

intramuscularly. Tests of antigenicity were determined both by microprecipitin and ring interface tests.

RESULTS.—*Virus concentration in various hosts.*—A comparative study with several systemic hosts demonstrated that PMV-M2 concentration (infectivity assay) was higher in Little Marvel pea than in peanut and other hosts (Fig. 1). Under greenhouse conditions (24 to 32 C), PMV-M2 infectivity in peanut and pea increased rapidly from inoculation time until the tenth day before it declined to a low level (25% of maximum peak) (Fig. 1). Since most of the infectivity was in the systemically infected leaves, this tissue was harvested from pea 9 to 12 days following inoculation.

Extraction medium and clarification.—Previous studies (19) established that PMV-M2 is nearly noninfective when diseased tissue is macerated without the presence of additives which affect oxidation reactions. Since infectivity was maintained better with phosphate buffer (pH 8.0) than acetate and borate (20), it was used as a general diluent. DIECA in combination with either ascorbic acid or NaHSO₃ (pH 7.2), was the best system for virus extraction. This extraction buffer gave two to three times more infective preparations than the phosphate buffer containing the following chemicals: NaHSO₃ - ascorbic acid (pH 5.2), cysteine HCl - Na₂SO₃ (pH 6.7), DIECA - Na₂SO₃ (pH 8.2), Na₂SO₃ - ascorbic acid (pH 6.7), or cysteine HCl - NaHSO₃ (pH 4.2). Infectivity was lost with a combination of cysteine HCl and ascorbic acid, probably due to a low pH (3.3).

Tests of several clarification procedures indicated that good clarification of PMV-M2 preparations caused extensive loss of infectivity. The best compromise method was a chloroform treatment (8 to 10%) which partially clarified and retained 50 to 60% infectivity. Freezing and low speed centrifugation gave more infective preparations but did not remove much of the chloroplast and other host materials. Other methods, e.g., heating the homogenate at 50 C, acidification to pH 5.0, charcoal treatment followed by filtration or centrifugation (8), magnesium-bentonite treatment (11), *n*-butanol-chloroform emulsification, 5 to 8% *n*-butanol treatment, and ethanol, caused major or total losses of infectivity.

Virus precipitation.—When chloroform-treated extracts were subjected to ultracentrifugation most of the jelly-like pellets usually did not go into solution regardless of the suspending media used. Hence, most of the virus was lost after subsequent low-speed centrifugation, suggesting that the virus was irreversibly aggregated. This contention was confirmed by the absence of a virus infectious zone following density-gradient centrifugation of the concentrated preparation. Ultrafiltration (23) was also tried as a means to concentrate the virus but it did not prevent aggregation.

The efficacy of PEG precipitation on PMV-M2 was examined with different concentrations and with different salts. The best combination was 4% PEG and

0.2 M KCl. This gave a relatively infectious preparation but did not prevent aggregation nor

remove all normal plant components. Furthermore, more than two precipitation steps with PEG drastically reduced infectivity. Virus recovery, however, was improved when the PEG precipitate was suspended in phosphate buffer containing 0.2 M urea or 0.001 M Cleland's reagent. Generally, the optical density readings at 260 nm of preparations suspended in urea were two times higher than those in Cleland's reagent. However, infectivity assays at various steps of purification revealed that virus treated with Cleland's reagent was usually two to four times more infective than urea-treated virus (Table 1).

Virus preparations suspended in Cleland's reagent exhibited an opalescent zone about 29 to 32 mm below the meniscus following density-gradient centrifugation. This band corresponded to an ultraviolet absorption peak obtained when the column was monitored through an ISCO fractionator and to the major portion of the infectivity in the column (Fig. 2A). Ultraviolet absorbance obtained at the top of the density-gradient column (Fig. 2A) was thought to be due to normal plant constituents since a similar reaction was also observed with preparations from healthy plants (Fig. 2C). In addition, no infectivity was found in the upper part of the column above the zone.

Virus was collected from the opalescent zone of general density-gradient tubes, concentrated by PEG precipitation, and subjected to a second density-gradient centrifugation. This virus preparation was free of the ultraviolet absorbing material at the top of the column and only one small peak was observed (Fig. 2B). About 50% of the total infectivity was lost during the second density-gradient run, and most of the lost infectivity could be accounted for in the pellet, which undoubtedly contained newly-aggregated virus particles. When pellets from density-gradient tubes were suspended in buffer containing Cleland's reagent and subjected to a second density-gradient run, aggregation was partially reversed as evidenced by a peak similar to that shown in Fig. 2B.

Characteristics of purified preparations.—Electron micrographs of virus preparations revealed flexuous rod particles (Fig. 3-A, B) and similar particles were not observed in healthy preparations. In the best preparations, particles were not aggregated and were substantially free from plant constituents. Measurements of 200 negatively stained particles

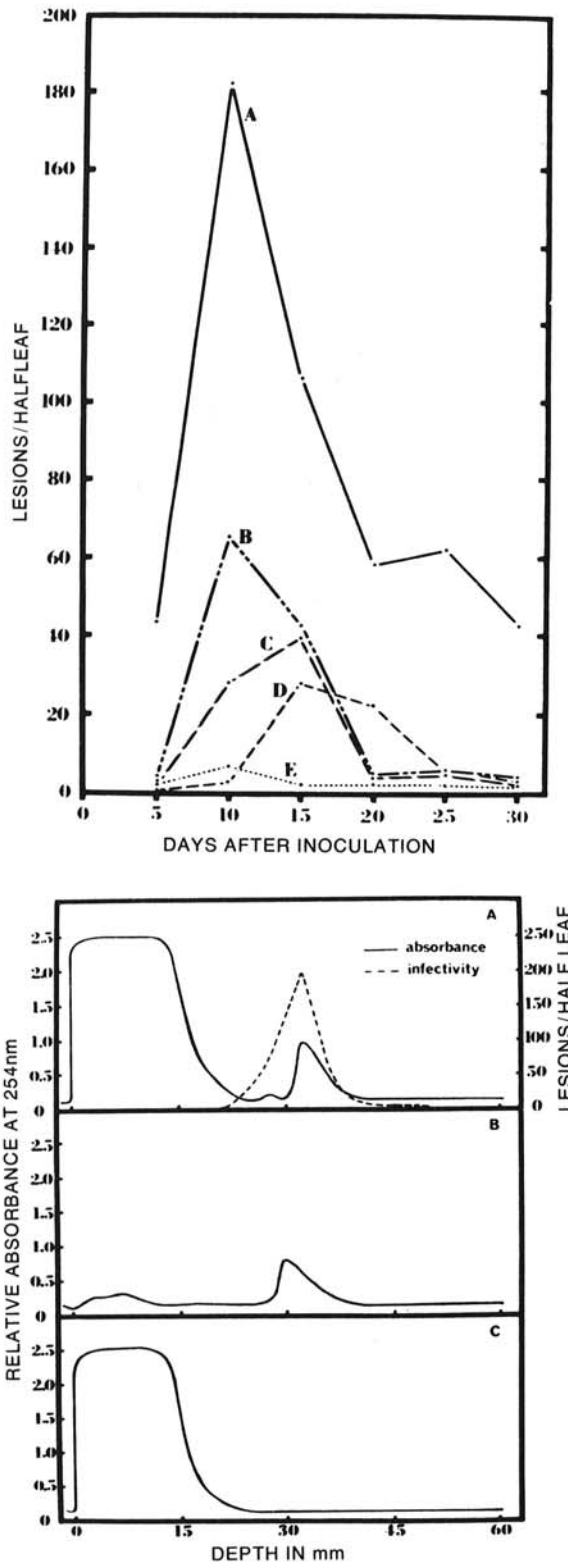


Fig. 1-2. 1) Local lesions produced on 'Topcrop' bean by sap from the following plants inoculated with a mild strain of peanut mottle virus (PMV-M2): A) 'Little Marvel' pea; B) 'Early Runner' peanut; C) 'Argentine peanut; D) 'Lee' soybean; and E) 'Early Ramshorn' cowpea. 2) Scanning patterns of PMV-M2 preparations in Cleland's reagent. A) PMV-M2 preparation after the first density-gradient centrifugation; B) PMV-M2 from zones of two density-gradient columns, concentrated with polyethylene glycol and subjected to a second density-gradient centrifugation; and C) healthy Little Marvel pea tissue prepared in the same manner as A.

showed particles of varying lengths. The most frequent length in the modal distribution of purified particles was 725 nm which is slightly shorter than the particle length (740 nm) obtained by the leaf dip method. The 725-nm particles represented 65% of the total particles measured. The shorter and longer particles constituted 24 and 11%, respectively. The diameter of the particles was approximately 12 nm.

The estimated yield of purified virus obtained from density-gradient zones was 1 to 2 mg/kg of tissue. This was based on the assumption that 3.0 OD units are equivalent to 1 mg/ml of virus, a common extinction coefficient for flexuous rod viruses (3). The absorption spectrum of purified virus was typical for nucleoproteins and a sample prepared similarly from healthy plants had low absorbance and no peak absorbance in the ultraviolet range. The spectrum showed minimum and maximum absorption at 246 nm and 260 nm, respectively. The 260:280 nm ratio was 1.24 and the 260:246 nm ratio was 1.13.

Serological test demonstrated that PMV-M2 was antigenic and a specific antiserum was produced. The titer was 1:256 and 1:128 with ring interface and microprecipitin tests, respectively. Normal rabbit serum and antiserum prepared against healthy plant constituents did not react with PMV-M2 fractionated from the density-gradient zone. Furthermore, partially purified and concentrated bean yellow mosaic and maize dwarf mosaic viruses, which have flexuous rod particles similar in length to PMV, did not react with PMV-M2 antiserum.

DISCUSSION.—The purification procedure adapted here gave a relatively pure virus preparation. A single zone was obtained in density-gradient columns, there was an absence of extraneous material in electron micrographs, and serological tests demonstrated a freedom from healthy plant constituents. The amount of virus obtained was 15 to 30 times less than the yield obtained with the severe mosaic strain of PMV (24). This difference could be explained by a relatively low concentration of PMV-M2 in pea or its high tendency to aggregate in vitro or both. Obviously, the 1 to 2 mg of virus/kg of plant tissue does not represent the amount of PMV-M2 in vivo because much virus is lost during purification.

The gross morphology of PMV-M2 suggests that this virus belongs to the potato virus Y group. The similarities of PMV to the group are as follows: (i) its particle length is within 720 to 800 nm (5), (ii) difficult to purify due to its tendency to aggregate and to be inactivated by most clarifying agents (5), and (iii) production of pinwheel and bundle-type inclusions (12, 14, 24). However, PMV has been found serologically unrelated to the following viruses: potato Y (24), tobacco etch (24), soybean mosaic (24), bean yellow mosaic, and maize dwarf mosaic.

Particle aggregation is one of the major problems in purification of flexuous rod type viruses. The formation of the aggregation is not understood. However, there is evidence that it is due to secondary forces such as hydrogen bonds, hydrophobic bonds, and electrostatic attractions of ionized groups and

TABLE 1. Effect of Cleland's reagent (0.001 M) and urea (0.2 M) on the infectivity of peanut mottle virus at different steps of purification of virus from 'Little Marvel' pea

Step of purification	Lesions/half leaf ^{a,b}	
	Cleland's reagent	Urea
First PEG ^c precipitation	67	18
Second PEG precipitation	26	12
Density gradient zone	7	8
Density gradient pellet	21	5

^a Before assay inoculation, each virus preparation was adjusted to an equal volume based on the original extraction.

^b Eight replications/treatment; LSD at 5% = 13; LSD at 1% = 18.

^c Polyethylene glycol 6000.

dipoles. The methods suggested to prevent aggregation include raising the pH, lowering the salt concentration, and addition of reducing agents. In the case of PMV-M2, these methods were not very effective.

Although the virus was successfully extracted in phosphate buffer containing DIECA and NaHSO₃ or ascorbic acid, this buffer and various antioxidants could not prevent or reverse aggregation in subsequent purification steps. Addition of DIECA and NaHSO₃ is believed to prevent inactivation of viruses by eliminating the copper necessary for

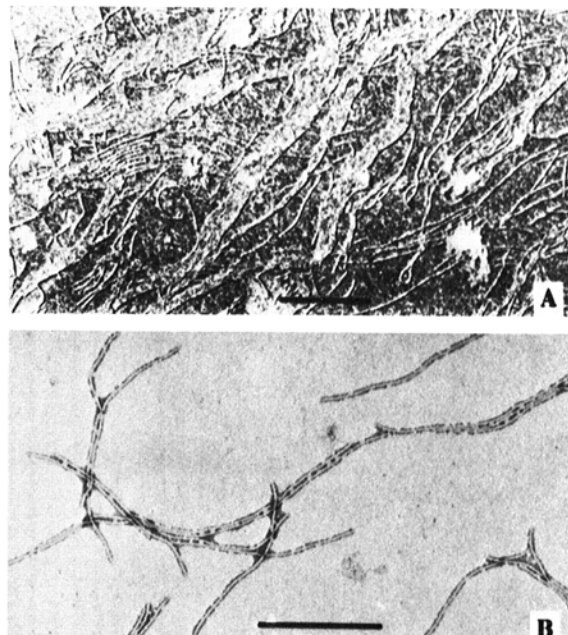


Fig. 3. Electron micrographs of a mild strain of peanut mottle virus particles from A) gold-palladium shadowed preparation of the first polyethylene glycol precipitation showing aggregated particles, and B) negatively stained (potassium phosphotungstate) particles from the zone of density-gradient columns. The scale represents 500 nm.

polyphenol oxidase activity, and general oxidation within the preparation, respectively.

Urea has been used successfully in preventing aggregation of several plant viruses because of its ability to break hydrophobic bonds (10). However, its effect on virus infectivity varied with the urea concentration. Urea at low concentrations (0.5 to 1.0 M) was reported to have no effect on tobacco etch virus infectivity but it may have reduced susceptibility of its host (10). Although urea partially prevented aggregation of PMV-M2, infectivity was substantially reduced despite the low concentration (0.2 M) used. Loss of infectivity could not be attributed to decreased host susceptibility, since preparations were dialyzed prior to inoculation.

Cleland's reagent (dithiothreitol) (6) is designed to protect sulfhydryl groups similar to the action of mercaptoethanol, cysteine, glutathione, or thioglycolate. However, Cleland's reagent is superior because it is less likely to be oxidized by air and because of its capacity to break disulfide bonds. Cleland's reagent has been used in dissolving protein materials (1, 9) and in dissociating virus particles into subunits (13, 16, 18). Its role in preventing and reversing virus aggregation, however, has not been explored. In view of the results obtained here, it appears that PMV-M2 particles may contain a substantial amount of SH groups. These groups could form into disulfide bonds during extraction and concentration steps and form virus aggregates. Addition of Cleland's reagent in the buffer could prevent or disrupt these bonds and allow the suspension of virus particles.

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