Peanut Science (1984) 11:40-42

An Efficient Procedure for Purification of an Isolate of Peanut Mottle Virus from Wild Peanut and Determination of Molecular Weights of the Viral Components[1]

John L. Sherwood[2]

ABSTRACT

An efficient procedure was developed for purification of peanut mottle virus (PMV) from pea (*Pisum sativum* cv. Little Marvel) that yielded 10-19 mg virus/ kg infected tissue. Virus was extracted from frozen infected tissue in 0.01 M potassium phosphate buffer, pH 8.0, with 0.001 M dithioerythritol, followed by clarification with chloroform (15%, v/v) and precipitation by KCl and polyethylene glycol. Virus was resuspended in 0.01 M borate-phosphate buffer, pH 8.3, with 0.2 M urea prior to density gradient centrifugation. Purified virus sedimented as a single component with a sedimentation coefficient of 149 S. The molecular weight of the single coat protein was estimated as 36,100 daltons in 12% polyacrylamide gels. The single nucleic acid isolated from PMV on sucrose gradients was degraded by RNase, but not DNase. The molecular weight of the RNA was estimted as 3.1×10^6 daltons on nondenaturing and denaturing sucrose gradients.

Key Words: *Arachis chacoense*, groundnut, PMV, potyvirus.

Peanut mottle virus (PMV), a potyvirus capable of infecting a number of legume species (6,7), is often difficult to purify because of aggregation of the virus particles. Until recently, procedures to purify the virus have yielded only a few mg of virus per kg of infected tissue (12). Tolin and Ford (16) reported a new method that yields 10-25 mg of virus per kg tissue, but only 200 g of tissue could be used in a purification. Thus, yields of only 2-5 mg per procedure are obtained. Rajeshwari et al. (14) reported a method that yields 30-40 mg of virus per kg tissue. Although good yields were obtained, their procedure is lengthy and is limited in the amount of tissue that can be processed in one purification because the virus preparation must be centrifuged through a sucrose cushion prior to density gradient centrifugation. Recently, Sanborn and Melouk (15) have identified and partially characterized PMV in a wild peanut (*Arachis chacoense* Krap. et Greg. nom. nudum, PI 276235). This paper describes an efficient procedure for processing up to a kg of virus infected fissue that yields 10-19 mg of virus per kg. In addition, the sedimentation coefficient, and molecular weights of the coat protein and nucleic acid of PMV from a wild peanut (PI 276235) are reported.

Materials and Methods

Virus source and assays. The isolate was from a single lesion obtained on *Phaseolus vulgaris* L. cv. Topcrop after transfer from a wild peanut, PI 276235, and subsequently maintained in *Pisum sativum* L. Alaska or Little Marvel (15). *P. sativum* cv. Little Marvel was used throughout these experiments because of its preferred growth habit. Inoculum was prepared by grinding leaf pieces in 0.01 M potassium phosphate buffer, pH 8.0, with 0.001 M dithioerythritol. Inoculations were made with small four-ply gauze pads saturated with inoculum and rubbed on leaves dusted with 225-μm corundum. Infectivity assays were determined by local lesion assays on *P. vulgaris* cv. Topcrop. Virus yeild was estimated spectrophotometrically assuming an A_{260} of 3.0 = 1 mg/mL (14).

Virus purification. PMV was purified by grinding frozen pea tissue harvested 11-14 days after inoculation in freshly prepared 0.01 M potassium phosphate buffer, pH 8.0, with 0.001 M dithioerythritol (2 mL/g) in a model CB-6 Waring blendor (New Hartford, CN 06057) at low speed. All subsequent steps were at 4 C unless noted. The tissue brei was filtered through cheesecloth and shaken with chloroform (15%, v/v) for 5 min. The emulsion was broken by centrifugation for 10 min at 10,000 *g*. Polyethylene glycol (PEG) (mol. wt. 8,000) and potassium chloride (KCl) were added to the supernatant, giving a final concentration of 4% and 1.5%, respectively. The mixture was stirred 30 min, let stand 2 hr, and centrifuged as above. The pellet from each 250 mL of centrifuged mixture was resuspended overnight in 30 mL of 0.01 M borate-phosphate buffer, pH 8.3, with 0.2 M urea. The resuspended pellets were collected, shaken with chloroform (15%, v/v) for 3 min and the emulsion centrifuged for 10 min at 10,000 *g*. PEG and KCl were added as above, the mixture stirred 20 min, let stand 2 hr, and centrifuged for 10 min at 10,000 *g*. The pellet was resuspended overnight in 0.01 M borate-phosphate buffer, pH 8.3, with 0.2 M urea (2.4 mL/100 g pea tissue). The resuspended pellets were collected and centrifuged for 5 min at 3,000 *g*. Four ml of the supernatant was placed on 10-40% sucrose gradients in 0.01 M potassium phosphate buffer, pH 8.0, with 0.001 M dithioerythritol. Density gradient centrifugation was carried out in a SW 25.1 Beckman rotor for 2.75 hr at 24,500 rpm at 14 C. Gradients were fractionated with an ISCO density gradient fractionator and UV analyzer (Lincoln, NE 68504). The single zone per tube was collected and centrifuged in a Beckman No. 30 rotor (Irvine, CA 92713) for 1.5 hr and pellets resuspended in 0.01 M potassium phosphate buffer, pH 8.0. Resuspended pellets were collected and centrifuged for 5 min at 3,000 *g* and the supernatant collected.

Sedimentation coefficient. The sedimentation coefficient of purified virus was determined by analytical ultracentrifugation. A 1.6 mg/mL sample was centrifuged at 32,000 rpm in the An-D rotor of the Beckman analytical ultracentrifuge with Schlieren optics. Sedimentation coefficients were determined using Markham's graphic method (11).

[1]Journal Article No. 4442, Oklahoma Agricultural Experiment Station, Oklahoma State University, Stillwater, OK.

[2]Assistant Professor, Department of Plant Pathology, Oklahoma State University, Stillwater, OK 74078.

Molecular weight determination of PMV protein and nucleic acid. Protein of PMV and molecular weight markers (Bio-Rad Laboratories No. 161-0304, Richmond, CA 94804) were prepared by heating virions and molecular weight markers at 95 C for 5 min in 0.0625 M Tris-HCl buffer, pH 6.8, with 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.002% Bromophenol Blue. Samples were then electrophoresed in a 12% polyacrylamide runnng gel (pH 8.8) with a 4% stacking gel (pH 6.8). Electrophoresis buffer contained 0.3% Tris, 1.44% glycine and 0.1% SDS, pH 8.3 (10). Electrophoresis was run in 1.5 mm slab gels at 20 mA until the tracking dye reached the running gel, and then at 30 mA until the tracking dye reached the bottom of the slab. Gels were stained in 0.5% Coomassie Blue in methanol-acetic acid-water (5:1:5), destained in methanol-acetic acid-water (50:7.5:42.5), and scanned at 590 nm using a Gilford Model 2600 spectrophotometer.

Viral nucleic acid of turnip mosaic virus (TuMV) and PMV was extracted as described by Brakke and van Pelt (2) using 0.2 M ammonium carbonate buffer, pH 9.0, with 0.002 M EDTA, 2% SDS and 400 μg/ml EDTA treated bentonite (8). TuMV was purified from *Brassica rapa* L. according to Choi et al. (13). The RNA was precipitated by ethanol after density gradient centrifugation. The molecular weight of PMV-RNA was determined by means of sedimentation of native and formaldehyde-treated RNAs by sucrose density gradient centrifugation. Formaldehyde treatment was conducted according to Choi et al. (4). TuMV-RNA (38.6 S = native form, 24.3 S = formaldehyde-treated) was used as a marker to determine the sedimentation coefficient of the native and formaldehyde-treated PMV-RNA. One ml of RNA was placed on 7.5-30% sucrose gradients in 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0, and centrifuged 14 hr at 22,000 rpm at 14 C in a Beckman SW 25.1 rotor. Gradients were fractionated as above. The molecular weight (M) of PMV-RNA was determined from the S value according to Hull et al. (9) and Brakke and van Pelt (2) using the formulas $M = 1550\ S^{2.1}$ for native RNA and $S = 0.083\ M^{0.38}$ for formaldehyde-treated RNA, respecitvely.

Results and Discussion

Host and extraction buffer. Maximum infectivity from systemically infected peas, as indicated by local lesion assay on Topcrop bean, was reached 10-14 days after inoculation. All experiments were conducted with material of that age. As others have found (14), phosphate buffer was found preferable to citrate or tris buffer. Material ground in 0.01 M phosphate buffer, pH 8.0, was more infectious than material ground in phosphate buffer at other concentrations (0.05, 0.1 or 1.0 M) or pH (7.0, 7.5 or 8.5). Dithoerythritol, 2-mercaptoethanol and Na_2SO_3 plus EDTA were tested as additives to retain the infectivity of PMV in phosphate buffer. Extraction buffer with 0.001 M dithioerythritol gave 1.5 to 2.5 times more infective preparations than extraction buffer with 0.75% or 1.5% 2-mercaptoethanol, or 0.02 M Na_2SO_3 plus 0.01 M EDTA.

Clarification. Freezing infected plant material 48 hr at -20 C prior to grinding had no deleterious effect on infectivity of the virus, but made tissue maceration easier. Frozen material retained 96-118% of the infectivity of non-frozen material. Clarification of macerated infected tissue by centrifugation alone was not satisfactory. When macerated tissue was shaken with different amounts of chloroform (v/v) for 5 min prior to centrifugation at 10,000 *g* for 10 min, clarification was achieved as well as an increase in infectivity. Macerated tissue mixed with chloroform at 10%, 20%, 50% or equal volumes was 102%, 130%, 125% and 120%, respectively, as infectious as tissue not centrifuged and not treated with chloroform. Tolin and Ford (16) also had found chloroform treatment did not adversely affect the virus.

Precipitation and resuspension. After clarification, virus was precipitated by adding PEG to a final concentration of 4% and either KCl or NaCl to a final concentration of 1.5%. KCl proved superior to NaCl for use in PMV purification. After precipitation with either salt and PEG, the virus was resuspended in 0.01 M potassium phosphate buffer, pH 8.0, with 0.5 M urea and 0.001 M dithioerythritol and subjected to density gradient centrifugation. Yields of virus from four trials using KCl compared to NaCl were 22-39% higher.

Two buffers were compared for resuspending the virus. A 0.01 M potassium phosphate buffer, pH 8.0, with 0.5 M urea and 0.001 M dithioerythritol; and a 0.01 M borate-phosphate buffer, pH 8.3, with 0.2 M urea (14). After the virus was precipitated with KCl and PEG it was resuspended with one of the two buffers. The borate-phosphate based buffer yielded 31-42% more virus based on results from 4 trials.

For PMV purification from small amounts of tissue, two other methods proved to be effective for concentrating the virus in lieu of precipitation by KCl and PEG after the second chloroform clarification. The addition of Lyphogel (Gelman Sciences, Inc., Ann Arbor, MI 48106) to the virus solution satisfactorily concentrated the virus for centrifugation in sucrose gradients in most cases. However, in some experiments the virus appeared to be absorbed in addition to the excess fluid as indicated by low yields from sucrose gradients. Dialysis of the virus soltuion against 10% PEG (mol. wt. 15,000 to 20,000) in 0.01 M potassium phosphate buffer, pH 8.0, with 0.001 M potassium dithioerythritol also was effective in concentrating the virus for density gradient centrifugation.

Yield and properties of purified virus. Yield from 5 separate 1 kg batches of infected tissue was 11.6, 13.5, 17.7, 9.5 and 18.6 mg (average = 14.2 mg/kg). Although these yields are similar to those previously reported (14,16), this method allows the processing of large amounts of tissue without a significant loss of virus. The difference in sensitivity of PMV from wild peanut to buffer and salt that have successfully been used to purify PMV isolated from cultivated peanut may reflect inherent differences in the isolates. Preparations had an absorbance maximum at 260 nm, an absorbance minimum at 246 nm and an inflection in the absorbance spectrum at 290 nm. The ratio of absorbance at 260 nm to 280 nm was 1.27, and the ratio of absorbance at 260 nm to 246 nm was 1.15. These values are similar to other PMV isolates (12,16). Purified virus preparations sedimented as a single component with a sedimentation coefficient of 149 S compared to 151 S for a PMV isolate from India (14). PMV purified by the procedure reported here reacted with PMV-specific antiserum provided by M. R. Sanborn, Oklahoma State University, in capillary ring interfacial tests and in enzyme-linked immunosorbent assay (5).

The molecular weight of the protein subunit, estimated from four runs by concurrent electrophoresis with standard protein markers, was 36,100 daltons (Fig. 1) compared to 34,000 dalton for a PMV isolate from India (14).

A single species of nucleic acid was isolated from PMV by density gradient centrifugation. When isolated PMV nucleic acid was treated with 5 μg/mL of pancreatic

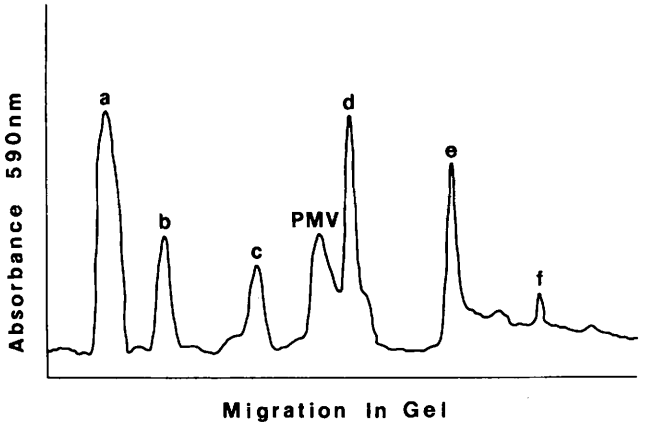

Fig. 1. Densitometer scan of 12% polyacrylamide gel of peanut mottle virus capsid protein (PMV) and molecular weight markers. Abreviations are: a) Phosphorylase B (92,500 daltons), b) Bovine serum albumin (66,200 daltons), c) Ovalbumin (45,000 daltons), d) Carbonic anhydrase (31,000 daltons), e) soybean trypsin inhibitor (21,500 daltons), and f) Lysozyme (14,400 daltons).

RNase and incubated at 37 C for 30 min prior to density gradient centrifugation, no peak was evident when the centrifuged gradients were fractionated. Similar treatment with 20 μg/ml DNase did not have this effect (Fig. 2).

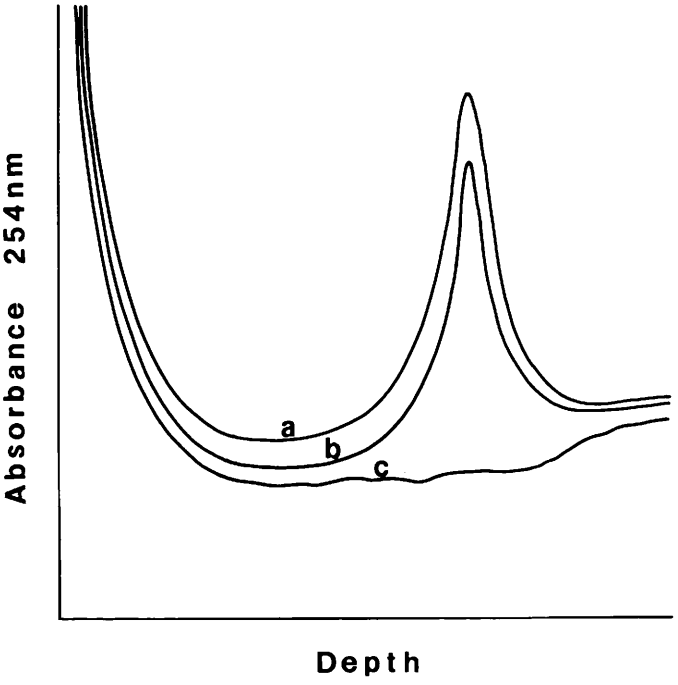

Fig. 2. UV-absorbance scanning profiles of peanut mottle virus (PMV) PNA preparations centrifuged in 7.5-30% sucrose gradients. Abbreviations are: a) native PMV-RNA, b) DNase-treated PMV-RNA, c) RNase-treated PMV-RNA.

When PMV-RNA was centrifuged concurrently with TuMV-RNA, PMV-RNA showed a sedimentation coefficient of 37.3 S. Using the formula of Hull et al. (9), the molecular weight of PMV-RNA was calculated as 3.1 x 10^6 daltons. When formaldehyde-treated PMV-RNA and formaldehyde treated TuMV-RNA were concurrently centrifuged, PMV-RNA showed a sedimentation coefficient of 24.3 S. Using the formula of Brakke and van Pelt (2), the molecular weight of PMV-RNA was calculated as 3.1 x 10^6 daltons which is the same as the RNA for a PMV isolate from India (14).

The procedure described in this paper permits purification of PMV from large amounts of tissue at one without substantial loss of the virus. The molecular weight of the nucleic acid of PMV from wild peanut appears similar to the isolate of Rajeshwari et al. (14). The difference in the molecular weight of the coat protein and sedimentation coefficient may reflect inherent physiochemical differences in the isolates. These appear to be the only two isolates of PMV for which these characteristics have been determined. Because a number of different isolates of PMV based on symptomology, host range, and aphid transmission have been identified (1, 13), a better determination of their relationships needs to be investigated.

Acknowledgment

I thank Dr. Howard Scott, University of Arkansas, Department of Plant Pathology, for determination of the sedimentation coefficient.

Literature Cited

1. Bays, D. C., and S. A. Tolin. 1983. Aphid transmissibility of five strains of peanut mottle virus. Phytopatholgy 73:787.
2. Brakke, M. K., and N. van Pelt. 1970. Properties of infectious ribonucleic acid from wheat streak mosaic virus. Virology 42:699-706.
3. Choi, J. K., T. Maeda and S. Wakimoto. 1977. An improved method for purification of turnip mosaic virus. Ann. Phytopath. Soc. Japan 43:440-448.
4. Choi, J. K., Matsuyama, N. and S. Wakimoto. 1979. Properties of ribonucleic acid isolated from turnip mosaic virus. 1. Determination of molecular weight. Ann. Phytopath. Soc. Japan 45:353-357.
5. Clark, M. F., and A. N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
6. Demski, J. W., Kahn, M. A., Wells, H. D. and J. D. Miller. 1981. Peanut mottle virus in forage legumes. Plant Dis. 65:359-362.
7. Demski, J. W., and C. W. Kuhn. 1975. Resistant and susceptible reaction of soybeans to peanut mottle virus. Phytopathology 65:95-99.
8. Fraenkel-Conrat, H., Singer, B., and A. Tsugita. 1961. Purification of RNA by means of bentonite. Virology 14:54-58.
9. Hull, R., Rees, M. W. and M. N. Short. 1969. Studies on alfalfa mosaic virus. 1. The protein and nucleic acid. Virology 37:404-415.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T_4. Nature 227:680-685.
11. Markham, R. 1960. A graphical method for the rapid determination of sedimentation coefficients. Biochem J. 77:516-519.
12. Paguio, O. R., and C. W. Kuhn. 1973. Purification of a mild mottle strain of peanut mottle virus. Phytopathology 63:720-724.
13. Paguio, O. R., and C. W. Kuhn. 1973. Strains of peanut mottle virus. Phytopathology 63:976-980.
14. Rajeshwari, R., Iizuka, N. Nolt, B. L. and D. V. R. Reddy. 1983. Purification, serology and physio-chemical properties of a peanut mottle virus isolate from India. Plant Pathology 32:197-205.
15. Sanborn, M. R., and H. A. Melouk. 1983. Isolation and characterization of mottle virus from wild peanut. Plant Dis. 67:819-821.
16. Tolin, S. A., and R. H. Ford. 1983. Purification and serology of peanut mottle virus. Phytopathology 73:899-903.

Accepted June 5, 1984