Etiology

Characterization of the Oat-Infecting Strain of Maize Dwarf Mosaic Virus

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ABSTRACT

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Virions of an oat-infecting strain of maize dwarf mosaic virus (MDMV-O) were purified using 0.01 M Tris-citrate, pH 7.0, by differential centrifugation, rate-zonal centrifugation in sucrose gradients, and isopycnic banding in CsCl gradients. Satisfactory virion resuspension after high-speed centrifugation also was obtained with 0.01 M potassium phosphate, pH 7.4, containing 1–3 M urea. Purified virions measured 650 to 808 × 14 nm and had an $s_{20,w}$ of 168 S and a buoyant density of 1.297 g/ml in CsCl. Viral nucleic acid was digested by RNase but not DNase, and virions contained 6.6% RNA by weight as determined by ultraviolet absorbance. Three virion capsid-protein subunits were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular masses (M_r) of the three protein subunits were estimated to be 35.1, 33.0, and 30.5 kDa. In contrast, single capsid-protein subunits were observed for virions of MDMV strains A, D, E, and F in SDS-PAGE, whereas three were observed for MDMV-B virions with M_r similar to those for the virion capsid-protein subunits of MDMV-O. Serologically, MDMV-O was distantly related to strains A, B, D, and F of MDMV as determined by enzyme-linked immunosorbent, immunodotblot, and microprecipitin assays, and to MDMV strain E and sugarcane mosaic virus strain A, but not H, as determined by the microprecipitin assay.

Recently, we reported some characteristics of a new strain of maize dwarf mosaic virus (MDMV), designated strain O, which was principally differentiated from other strains by its ability to infect oats (*Avena sativa* L.) (30). The virus was identified as a strain of MDMV by its host range, which included johnsongrass (*Sorghum halepense* (L.) Pers.) and maize (*Zea mays* L.); the flexuous morphology and 740-nm modal length of its virion; the combination of nonpersistent transmission by aphids and mechanical transmission; and its association in infected maize cells with cylindrical inclusions containing pinwheels and scrolls but no laminate aggregates. However, MDMV-O failed to react with antisera to MDMV strains A and B in microprecipitin, double antibody sandwich enzyme-linked immunosorbent (DAS-ELISA), and agar-gel double diffusion assays. These findings prompted us to characterize MDMV-O further, particularly its relationship to other MDMV strains.

MATERIALS AND METHODS

Virus isolates. MDMV strains A, B, D, E, and F were maintained and characterized at Ohio (29); MDMV-O was obtained from Texas (30); and sugarcane mosaic virus (SCMV) strains A and H were from Texas. Viruses were maintained and inoculations were made as described previously (30).

Tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV), isolated from greenhouse-grown tomato (*Lycopersicon esculentum* L.) and cucumber (*Cucumis sativus* L.), respectively (D. T. Gordon, *unpublished*), were maintained in tobacco (*Nicotiana tabacum* L. 'F_2C_1').

Purification. In the first of two purification protocols, MDMV-O-infected Oh28 maize leaf tissue with pronounced symptoms was harvested 2–3 wk after inoculation, and 70-g samples were homogenized in five volumes of 0.01 M Tris-0.01 M citrate buffer, pH 7.0, (Tris-citrate buffer) plus 0.5% 2-mercaptoethanol. The extract was expressed through cheesecloth and then emulsified with one-fourth volume chloroform. The emulsion was separated

by centrifugation at 10,000 rpm in a Beckman JA-14 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 10 min, and the aqueous phase was recovered and poured through polyester fiber. Virions were pelleted through a layer (6.2 ml) of 30% sucrose in Tris-citrate buffer by centrifugation at 30,000 rpm in a Beckman Type 35 rotor for 90 min. The pellets were suspended in Tris-citrate buffer (1 ml/20 g of extracted tissue), the suspension was emulsified with chloroform as above, and the aqueous phase was recovered after centrifugation at 10,000 rpm in a Beckman SW50.1 rotor for 10 min. The aqueous phase was layered onto linear 10–40% sucrose density gradients prepared in Beckman SW28 rotor tubes using 7-, 10-, 10-, and 5-ml layers of 400, 300, 200, and 100 mg of sucrose/1 ml of Tris-citrate buffer, respectively. Gradients were centrifuged at 26,000 rpm at 15 C for 150 min, and the virion-containing band was collected using an ISCO Model 640 Density Gradient Fractionator and Model UA-5 Absorbance Monitor (Instrument Specialties Co., Lincoln, NB). Virion fractions were diluted with Tris-citrate buffer and centrifuged at 30,000 rpm in a Type 35 rotor for 90 min, and the pellets were suspended in 3.5 ml of Tris-citrate buffer to give partially purified MDMV-O.

An assay to test the infectivity of partially purified MDMV-O was conducted on Oh28 maize as described by Brakke (4). Virion yields were estimated with a Beckman Acta V spectrophotometer using an extinction coefficient of 2.7 $(mg/ml)^{-1}$ cm^{-1} at 260 nm as determined for MDMV-B (26).

In the second purification protocol, MDMV-O virions were partially purified from systemically infected Oh28 maize leaves, harvested 2–5 wk after seedling inoculation, as described previously (30). Different molarities (0.0, 0.01, 0.05, 0.1, 0.5, and 1.0 M) of phosphate buffer and of urea (0.1, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 M) in 0.01 M potassium phosphate, pH 7.0, were tested for their beneficial effect on virion resuspension and prevention of virion aggregation following the initial high-speed centrifugation. The relative amount of resuspended virion and the degree of virion aggregation were assessed by rate-zonal centrifugation on sucrose density gradients as described in the following section.

Published procedures were followed to purify CMV (28) and TMV (1).

Rate-zonal centrifugation assay. Assays were done using sucrose gradients prepared in Beckman SW50.1 rotor tubes. For the phosphate molarity series, 5–35% sucrose in the same molarity of phosphate as the resuspension buffer was used; for the urea molarity series, 10–40% sucrose in phosphate-urea of the same molarity as the resuspension buffer was used. Suspensions of partially purified virions (0.1 or 0.2 ml) were layered on gradients. After centrifugation to $5.25–5.35 \times 10^{10}$ rad^2/sec (almost 50 min) at 45,000 rpm, gradients were scanned at 254 nm, and the area under the virion ultraviolet-absorbance peak was measured. The area measurements were used as the relative virion concentrations as described previously (11).

Electron microscopy. To estimate particle dimensions, partially purified MDMV-O virions in Tris-citrate buffer were stained with neutralized 3% potassium phosphotungstate (PTA) and examined with the electron microscope.

To estimate the effect of various preparative procedures on particle dimensions, partially purified virions were treated with 1% glutaraldehyde and then stained with PTA on Formvar-coated grids as described above or shadowed using either a carbon/platinum or gold-palladium wire in a Kinney Vacuum Evaporator (Kinney Vacuum Division, Boston, MA) and Ladd electrodes (Ladd Research Industries, Inc., Burlington, VT) at an angle of 20° and a distance of 5 cm from the grid surface. Partially purified TMV virions were added as an internal measurement standard. Measurements were made as previously described (30) using an Electronics Graphics Calculator (Numonics Corp., Lansdale, PA) from 3× prints of particles magnified 10,000×. The microscope was routinely calibrated at 10,000× and 45,000× using fragments of a 2,160-line-per-nanometer, crossed-lined grating replica (Ernest F. Fullam, Inc., Schenectady, NY).

Serology. Preparation or source of antisera. For MDMV-O, preimmune serum was collected from a New Zealand white rabbit which was subsequently injected intramuscularly with about 20–50 μg of purified virion emulsified in an equal volume of Freund's complete adjuvant at 5–6-wk intervals for a 5-mo period. Antiserum used in tests was collected about 7 days after the last injection and had a homologous titer of 1/128 in the microprecipitin assay. The antiserum was mixed with an equal volume of glycerol and stored at −23 C. Antisera to MDMV strains A, B, D, and F were prepared previously (D. T. Gordon, *unpublished*; 24). Antisera to a Louisiana isolate of MDMV-A and to strains H and I of SCMV from Louisiana were provided by A. G. Gillaspie, Jr.

Microprecipitin assay. The assay was performed as described previously (11,24), except that plates were incubated for about 12 hr at 3 C and at room temperature for another 12 hr and then assay reactions were recorded. Viral antigens were either partially purified virion preparations or phosphate-buffered saline (0.02 M sodium phosphate, pH 7.4, plus 0.15 M NaCl) extracts from infected maize (inbred Oh28) or sorghum (*Sorghum biocolor* (L.) Moench 'Sart').

Enzyme-linked immunosorbent assay. An indirect DAS-ELISA was performed using polystyrene microtitration "U" plates (Dynatech Laboratories, Inc., Alexandria, VA). Coating antibodies were $F(ab')_2$ fragments prepared from IgGs to MDMV strains A, B, D, F, and O. IgG fractions were purified from 1.0 ml of anti-MDMV serum diluted first with an equal volume of glycerol and then in an equal volume of 20 mM Tris-Cl, pH 8.0, plus 0.1 M NaCl. This diluted antiserum was passed through a 5.0-ml protein A-Sepharose column (Sigma Chemical Co., St. Louis, MO) in Tris-Cl buffer. The column was washed with six volumes of Tris-Cl buffer, and bound IgG was eluted with 0.1 M glycine-Cl, pH 3.0. The eluate (500 μl/fraction) was collected in tubes containing 50 μl of 1.0 M Tris. The protein concentration of the fractions was determined by the Bio-Rad Colorimetric Protein Assay (Bio-Rad Laboratories, Richmond, CA). One milliliter of the pooled protein-containing fractions was dialyzed overnight against 500 ml of digestion buffer (0.07 M sodium acetate, pH 4.0, plus 0.05 M NaCl (3). Pepsin (2,500–3,200 units/mg of protein) (Sigma Chemical Co.) was added to 45 μg/ml of IgG dialyzed fraction, and the mixture was incubated at 37 C on a rocker shaker for 18 hr. The resulting $F(ab')_2$ units were desalted on a 7.5-ml G25 column (Pharmacia Fine Chemicals, Piscataway, NJ). Sodium azide was added to the pooled protein-containing fractions. In preliminary tests, the following amounts of reagents gave optimum ELISA results when tested with the appropriate homologous antigen concentration: 5 μg $F(ab')_2$/ml of 0.05 M sodium carbonate buffer, pH 9.6, for coating plate wells; 5 μg of purified IgG/ml of Tris-buffered saline plus Tween 20 (TBS-T) (250 mM Tris-Cl, pH 8.0, 0.15 M NaCl, and 0.05% Tween 20 [Sigma Chemical Co.]) for detecting antibody; and a 1:1,500 dilution of alkaline phosphatase conjugated protein A (Sigma Chemical Co.) in TBS-T. In cross-reactivity tests, purified virions of MDMV strains were diluted to 10 g/ml of PBS-T (0.02 M sodium phosphate buffer, pH 7.4, 0.15 M NaCl, and 0.05% Tween 20) and then tested by indirect DAS-ELISA as described below. The volume of reagents and virion suspensions added to each well was 50 μl, except as noted. Six replicates per experiment were tested for each strain.

Indirect DAS-ELISA wells were coated with $F(ab')_2$ fragments (0.1 ml/well) overnight at 4 C and then washed three times with PBS-T. The antigen was added and the plates were incubated for 2 hr at 37 C. Wells were washed as before, and IgG was added and incubated for 2 hr at 37 C. After three washes with TBS-T, protein A conjugate was added and incubated for 2 hr at 37 C. After two washes each with PBS-T and TBS-T, respectively, 75 μl of *p*-nitrophenyl phosphate (Sigma Chemical Co.) (1.0 mg/ml 10% diethanolamine, pH 9.8) was added per well and the plates were incubated at room temperature until the A_{405} of homologous antibody-antigen mixtures reached 1.0. Reactions were terminated by adding 30 μl of 3 M NaOH/well. Absorbances of well contents were measured as previously described (30).

Immunodotblot assay. This assay was performed by spotting 2 μl of diluted, purified-virion suspensions onto nitrocellulose membrane strips (Millipore Corporation, Bedford, MA). The strips were blocked with TBS-T plus 5% nonfat dry milk for 30 min

at 37 C. Then IgG to each MDMV strain, diluted 1:200 in TBS-T containing 1% gamma globulin-free horse serum, was incubated with strips for 1.5 hr at 37 C. The strips were washed once with TBS-T plus 1% horse serum for 10 min and twice with TBS-T, 5 min per wash. The protein A conjugate was diluted 1:50 in TBS-T plus 1% horse serum and incubated with strips for 1.5 hr at 37 C. Strips were washed twice with TBS-T plus 1% horse serum, two times with TBS-T, and once with TBS, 5 min per wash. The strips were laid, antigen side down, on a 1% agarose gel containing 0.1 mg of 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.) per milliliter of buffer. The agarose was dissolved in a 0.1 M Tris-Cl, pH 8.3, buffer plus 1 mM $MgCl_2$. Strips were covered with plastic wrap and incubated at room temperature overnight (23). Reactions were considered positive when blue dots were present on both the nitrocellulose strip and the matching area of the gel.

Sedimentation coefficient. The sedimentation rate of MDMV-O virions was determined in linear-log gradients following a procedure described previously (9), except that the centrifugation was at 6 C and 40,000 rpm (Beckman SW 41 Ti rotor) for intervals of 4.0, 6.0, or 8.0×10^{10} rad^2/sec (30, 40, or 60 min, respectively).

Buoyant density. Solid CsCl was added to partially purified MDMV-O virions suspended in Tris-citrate buffer to give a density of 1.300 g/ml. The mixture was centrifuged in a Beckman SW50.1 rotor at 39,000 rpm and 10 C for $1.18–1.25 \times 10^{12}$ rad^2/sec (overnight). The MDMV-O virion-containing band was collected, its refractive index (RI) was determined with a Bausch and Lomb Abbe 3-L Refractometer (Bausch & Lomb, Inc., Rochester, NY), and its density was estimated from an RI versus density (CsCl in Tris-citrate buffer) plot. Partially purified MDMV-O virions were sometimes further purified through CsCl gradients to give purified virions.

Nucleic acid. Nucleic acid was released from purified MDMV-O virions by heating to 60 C for 8 min in 1% sodium dodecyl sulfate (SDS) in Tris-citrate buffer. The nucleic acid was partially purified on 5–20% linear RNase-free sucrose density gradients in Beckman SW50.1 tubes centrifuged at 40,000 rpm and 6 C for 1.9×10^{11} rad^2/sec (3 hr). The nucleic acid was recovered, dialyzed against Tris-citrate buffer containing 1 mM magnesium as $MgCl_2$, and incubated with equal volume of either RNase A (5 μg/ml) or DNase I (5 μg/ml) (Worthington Biochemical Corp., Freehold, NJ) for 30 min at 35 C. Samples then were rerun on sucrose gradients.

The percentage of RNA in purified MDMV-O virions was estimated using the Gibbs and Harrison (7) plot of absorbance ratio A_{260}/A_{280} versus percentage of RNA.

Viral capsid protein. The molecular masses (M_r) of viral capsid-protein subunits were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 8, 10, and 12% slab gels (14× 16 cm, 1.5-mm thick) using the Laemmli (25) system. Virions or M_r standard proteins in the Tris-citrate buffer were combined with an equal volume of treatment buffer (0.125 M Tris-Cl, pH 6.8, plus 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol). The mixture was heated for 90 sec in boiling water to degrade the virions. The mixture was cooled on ice, and then a bromophenol blue tracking dye-sucrose solution was added. MDMV-O capsid-protein subunits and standard proteins also were reduced and carboxymethylated as described previously (5), and the virions were dissociated to give coat-protein subunits as above. Samples were layered onto a 4% acrylamide stacking gel and electrophoresed at 15 mA constant current until the tracking dye reached the separating gel, and then the current was increased to 20 mA. Slab gels were kept at 11–12 C during the 6-hr run in a water-cooled Hoefer SE600 electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA).

Gels were stained at room temperature for 5–8 hr in 0.125% Coomassie Blue R-250 in a 50% methanol-10% acetic acid solution. Gels were destained with 50% methanol-10% acetic acid followed by 5% methanol-7% acetic acid in a Bio-Rad Gel Electrophoresis Slab Diffusion Destainer, Model 222 (Bio-Rad Laboratories).

The M_r of noncarboxymethylated capsid-protein subunits was calculated as previously described (6,13), whereas the M_r of carboxymethylated capsid-protein subunits was computed from plots of R_f of the reduced, carboxymethylated standard proteins versus their log molecular weight (42). The M_r-standard proteins (Sigma Chemical Co.) were bovine serum albumin (68.0 kilodaltons [kDa]), chymotrypsinogen (25.0 kDa), and RNase A (13.7 kDa).

In tests of the influence of host plant species on capsid-protein subunit characteristics, chloroform-clarified extracts from infected leaves of Oh28 maize, Garland oats, and Sart sorghum were analyzed by SDS-PAGE. Migration distances (mm) of MDMV-O capsid-protein subunits were obtained by scanning (bottom to top of gel) photographic negatives of gels with a Beckman Model R-110 Microzone Densitometer at 520 nm.

To test the stability of MDMV-O capsid protein, purified virions were degraded to yield subunits which were stored with added sodium azide at 3 C for up to 66 days. Aliquots were withdrawn from the preparation at 11-day intervals and electrophoresed in SDS-10% polyacrylamide gels.

RESULTS

Virion purification. After centrifugation, partially purified preparations of MDMV-O gave a single, opalescent, ultraviolet-absorbing band in sucrose gradients (Fig. 1). Purified MDMV-O virions had a maximum absorbance at 260 nm, a minimum near 245 nm, and an A_{260}/A_{280}, corrected for light scattering, of 1.28 (standard deviation [SD] = 0.018, number of tests [N] = 12). Partially purified MDMV-O virion preparations contained flexuous, filamentous particles of fairly uniform length and few contaminants. The average yield from six partially purified preparations was 31 μg (SD = 7.91) virion/gram of infected tissue. Virion loss probably due to virion aggregation was evident in that virion-containing pellets were found after centrifugation in sucrose gradient tubes.

Partially purified MDMV-O virions at decreasing concentrations gave the following infectivity ratios (number of symptomatic maize seedlings per number of inoculated seedlings): 250 μg/ml, 30/35; 50 μg/ml, 8/34; 25 μg/ml, 5/29; 5 μg/ml, 0/31; 2.5 μg/ml, 1/35; and 0.5 μg/ml, 0/36. These responses demonstrated that the partially purified virions were infective and that numbers of infected seedlings were directly related to virion concentration.

Among the various molarities of pH 7.0 potassium phosphate buffers tested for resuspension of partially purified MDMV-O virions, 0.01 M was the most effective. The average relative amounts (%) of virions banded in centrifuged sucrose gradients in the four tests were 49, 100, 77, 20, 15, and 0 for virions suspended and centrifuged in distilled water and 0.01, 0.05, 0.1, 0.5, and 1.0 M phosphate, respectively. Addition of 1–3 M urea to the 0.01 M

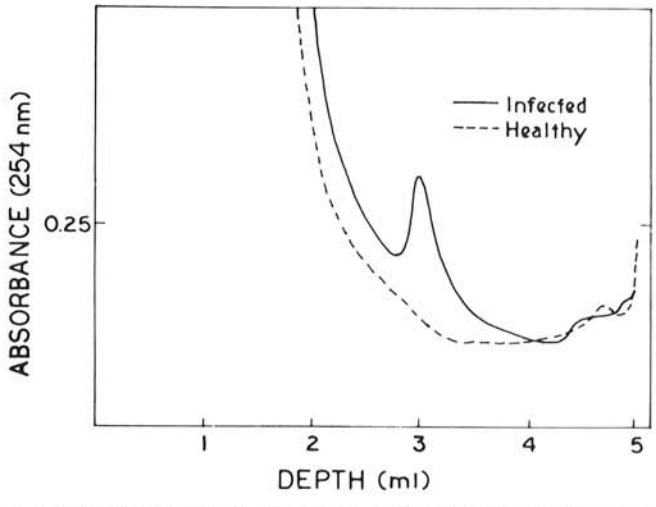

Fig. 1. Ultraviolet (254 nm) absorbance profile of a centrifuged sucrose density gradient containing a preparation of maize dwarf mosaic virus strain O from infected Oh28 maize (*Zea mays*). The virions had been concentrated previously by high-speed centrifugation.

potassium phosphate, pH 7.0, increased the amount of virions four- to fivefold. Higher concentrations of urea eliminated virion bands from the centrifuged gradients.

Electron microscopy. Shadow-cast, partially purified MDMV-O virions had a mean length of 765 nm (SD = 218, N = 125) with 71% of the particles between 700 and 800 nm in length. The mean and median lengths of nonglutaraldehyde-treated, PTA-stained particles were 695 (SD = 151, N = 83) and 699 nm (N = 83), respectively. The particle diameter was 14 nm (SD = 1.0, N = 83). The internal standard TMV particles had a mean and median particle length of 321 (SD = 13.4, N = 100) and 272 nm (N = 100), respectively, indicating a 7 and 10% error in the mean and median measurements, respectively, for the untreated MDMV-O particles, assuming a TMV particle length of 300 nm.

In a separate determination of length, the mean and median lengths for glutaraldehyde-treated and untreated MDMV-O particles stained with PTA were 808 (SD = 123, N = 50) and 728 nm, and 670 (SD = 79, N = 50) and 688 nm, respectively, indicating that, based on mean and medium lengths, untreated particles were 17 and 5% shorter, respectively, than glutaraldehyde-treated particles.

Serology. Microprecipitin assay reactions of MDMV-O and the other MDMV and SCMV strains in infected plant extracts are summarized in Table 1. MDMV-O antiserum reacted with all the MDMV strains and SCMV-A but not SCMV-H, whereas MDMV-O reacted with antisera to MDMV strains A (Ohio), O, and D and SCMV-H but not with those to another isolate of MDMV-A or MDMV-B and MDMV-F, and SCMV-I.

Also in the microprecipitin assay, partially purified MDMV-O virions (350 μg/ml) gave trace reactions (based on relative amount of precipitate) with MDMV-A and MDMV-B antisera, whereas MDMV-O antiserum gave intermediate reactions with MDMV-A (330 μg/ml) and MDMV-B (200 μg/ml) virions. Homologous reactions gave the best responses, that is, the greatest amount of precipitate.

In ELISA (Table 2) and immunodotblot assays (Table 3), MDMV-A, -D, and -F were closely related to each other; MDMV-B was moderately related to MDMV-A, -D, and -F; and MDMV-O was distantly related, if at all, to the other strains.

Sedimentation coefficient. The $s_{20,w}$ of MDMV-O was 168 S (SD = 8.66, N = 14). For the reference particle (CMV), the plot of distance sedimented, as measured on records of ultraviolet-absorbance profiles of centrifuged gradients, versus centrifugal force (w^2t) had an $R^2 = 0.964$ for N = 14, indicating a linear relationship between the two parameters. The equation of the plot was used to determine the $s_{20,w}$ for MDMV-O virions as described previously (9).

Buoyant density. MDMV-O virions formed a discrete band at 1.297 g/ml (SD = 0.003, N = 12) after overnight centrifugation in gradients of CsCl in Tris-citrate buffer, pH 7.0.

Nucleic acid. Nucleic acid treated with RNase A yielded slowly sedimenting ultraviolet-absorbing material, presumably a degradation product of the MDMV-O virion nucleic acid that was not seen after DNase I treatment (Fig. 2). The latter treatment had no effect on the banding characteristics of MDMV-O virion nucleic acid. The weight percentage of RNA in MDMV-O virions was estimated to be 6.60 (SD = 0.301, N = 15).

Viral capsid protein. MDMV-O capsid protein obtained from purified virion preparations migrated as three bands in SDS-polyacrylamide gels (Figs. 3A and 4). The top (T) and middle (M)

TABLE 1. Serological relationships among maize dwarf mosaic virus (MDMV) strain O and other MDMV and sugarcane mosaic virus (SCMV) strains as demonstrated by the microprecipitin test

	Antigens[a]									
	MDMV								SCMV	
Antisera	O	A	A[b]	B	B[b]	D	E	F	A[b]	H[b]
MDMV-O	3[c]	2	1	1	1	1	1	1	1	0
MDMV-A(OH)	1	3	1	1	1	2	2	2	1	0
MDMV-A(LA)[b]	0	1	2	0	0	1	1	2	0	0
MDMV-B	0	0	0	1	1	0	0	0	1	0
MDMV-D	1	2	2	0	1	2	1	2	1	0
MDMV-F	0	2	2	0	1	1	1	2	1	0
SCMV-H[b]	1	1	1	1	1	1	1	1	2	1
SCMV-I[b]	0	0	0	0	0	1	1	1	0	1

[a] Antigens prepared by grinding infected leaf tissue 1:2, w/v, in phosphate-buffered saline plus sodium azide, pH 7.0, and partial clarification by low-speed centrifugation.
[b] Source: P. Berger (antigens); A. J. Gillaspie, Jr. (antisera).
[c] Amount of precipitate: 0 = none; 1 = trace; 2 = intermediate; 3 = abundant.

TABLE 2. Serological reactivity of purified maize dwarf mosaic virus (MDMV) strains A, B, D, F, and O with their respective antibodies as determined by the enzyme-linked immunosorbent assay (ELISA)[a]

Antigen[b]	Experiment no./reactivity (%)[c]	Antibodies to:				
		MDMV-A	MDMV-B	MDMV-D	MDMV-F	MDMV-O
MDMV-A	1	1.004 ± 0.026[d]	0.275 ± 0.007	0.915 ± 0.018	1.152 ± 0.002	0.076 ± 0.018
	2	1.076 ± 0.020	0.3195 ± 0.061	0.990 ± 0.024	1.020 ± 0.011	0.0545 ± 0.001
	%	100	30	93	95	6.5
MDMV-B	1	0.027 ± 0.018	0.978 ± 0.053	0.058 ± 0.020	0.079 ± 0.006	0.010 ± 0.005
	2	0.034 ± 0.006	0.996 ± 0.054	0.048 ± 0.011	0.0745 ± 0.004	0.003 ± 0.003
	%	3	100	5	7	0.6
MDMV-D	1	0.818 ± 0.051	0.153 ± 0.015	1.009 ± 0.020	1.1795 ± 0.027	0.080 ± 0.019
	2	0.939 ± 0.021	0.202 ± 0.017	1.034 ± 0.010	1.013 ± 0.039	0.082 ± 0.005
	%	84.5	18	100	96	8
MDMV-F	1	0.656 ± 0.070	0.1375 ± 0.012	0.7095 ± 0.036	1.215 ± 0.082	0.021 ± 0.004
	2	0.861 ± 0.008	0.193 ± 0.014	0.832 ± 0.061	1.0625 ± 0.023	0.024 ± 0.003
	%	73	17	75.5	100	2
MDMV-O	1	−0.002 ± 0.003	−0.019 ± 0.004	0.006 ± 0.006	0.084 ± 0.006	0.995 ± 0.013
	2	0.002 ± 0.002	−0.0025 ± 0.003	0.0065 ± 0.005	0.045 ± 0.006	1.020 ± 0.039
	%	0	0	0.6	6	100

[a] A modified indirect double antibody sandwich ELISA was performed. Coating antibodies were F(ab')$_2$ fragments (5 μg/ml); detecting antibodies were purified IgG (5 μg/ml), enzyme conjugate was protein A conjugated with alkaline phosphatase (1:1500); and the substrate was *p*-nitrophenyl phosphate (1.0 mg/ml of 10% diethanolamine). Reactions were stopped with 3 M NaOH.
[b] The viral antigens were purified MDMV strains diluted to 10 μg/ml.
[c] % denotes the mean A_{405nm} for the specific virus-antibody test of two experiments as the numerator and the mean A_{405nm} of the homologous reaction for the same antibodies as the denominator. The dividend was expressed as a percent.
[d] The mean A_{405nm} and standard deviation for six replicates per antigen-antibody combination per experiment. The mean A_{405nm} of the corresponding negative controls, healthy maize leaf extracts in phosphate-buffered saline plus Tween 20, was subtracted from each mean A_{405nm}. The A_{405nm} for these negative controls ranged from 0.008 to 0.019.

bands were large and distinct, whereas the bottom (B) band varied in size and distinctness, being absent or barely visible in some PAGE runs. Reduced, carboxymethylated MDMV-O capsid protein also migrated as three bands (Fig. 4), but each had a higher molecular weight than the corresponding band for the capsid protein not reduced and carboxymethylated. For MDMV-O from clarified extracts from maize and sorghum, the migration distances of the two larger capsid-protein subunits (T and M) were similar. The fastest migrating band (B) was discernible only for the capsid protein from MDMV-O-infected sorghum. The relative migration distances (mm), obtained from a densitometer scan (from bottom to top) of electrophoresed, stained gels, were for maize T = 17.9 and M = 15.5 and for sorghum T = 18.1 and M = 15.5. The relative migration distances for these two capsid-protein subunits from MDMV-O-infected oats were slightly greater with T = 19.6 and M = 16.1.

The M_rs of the noncarboxymethylated capsid-protein subunits were estimated to be 35.1 (T), 33.0 (M), and 30.5 (B) kDa. The M_r of the MDMV-A capsid-protein subunit (Fig. 4) was 27.7 kDa (N = 15), and those of the MDMV-B capsid-protein subunits (Fig. 4) were 34.7 (T), 33.0 (M), and 30.8 (B) kDa for the three bands (in Fig. 4, the 30.8 kDa subunit is missing). Capsid-protein subunits of MDMV strains D, E, and F migrated as single bands indistinguishable from each other in migration distance (Fig. 5).

For the reduced, carboxymethylated MDMV-O capsid-protein subunits electrophoresed in 10% polyacrylamide gels, the M_rs were 46.2 (T) and 45.9 (M) kDa (N = 11). The bottom protein band was too indistinct for accurate measurement of its migration distance.

In the test of MDMV-O capsid-protein stability, the three main capsid-protein bands were present in preparations stored less than 34 days (Fig. 3a), although the middle protein band appeared as a doublet in some runs (data not shown). Additional bands, presumably degradation products of the three bands, were revealed by SDS-PAGE of capsid-protein subunits stored for 44–66 days (Fig. 3b).

DISCUSSION

The purification procedure using Tris-citrate buffer gave infective MDMV-O relatively free of host contaminants as judged by ultraviolet-absorbance characteristics, SDS-PAGE, and transmission electron microscopy. Amounts of partially purified MDMV-O virions (31 μg/g of infected maize leaves) were comparable to the 5–28 μg/g reported by Langenberg (26) for MDMV-B also extracted and resuspended in a Tris-citrate buffer and the 15–30 μg/g reported by Tolin and Ford (40) for various MDMV strains.

Previously, we (30) purified MDMV-O virions using 0.5 M potassium phosphate, pH 7.0, as both the extraction and resuspension buffers. However, use of this buffer for resuspension of virions in pellets from high-speed centrifugation did not give consistently satisfactory suspensions (D. T. Gordon, *unpublished*). In the present study, 0.01 M was the optimal concentration of phosphate for virion resuspension, and incorporation of 1–3 M urea further improved resuspension. Urea has been used to advantage previously in potyvirus, including MDMV, purification (5,11,18) and presumably reduces virion aggregation.

The MDMV-O particle length (650–808 nm) was similar to values reported by Shepherd (37), Bancroft et al (2), Sehgal (35) and Thornberry and Phillippe (39) for other MDMV isolates. Our estimate of 14 nm as the particle diameter corresponds to the 12–15 nm reported by Sehgal (36). The length of shadow-cast and glutaraldehyde-treated MDMV-O particles was greater than that of PTA-stained ones, suggesting that the first two treatments produced less particle fragmentation of purified virions than did PTA. We suggest that the longer lengths of the shadow-cast and glutaraldehyde-treated particles are representative of the lengths of unaltered particles. This also is suggested by our previous (30) finding that the mean and median lengths of particles from leaf-dip preparations were 801 (N = 17) and 722 nm, respectively. Particles from these leaf-dip preparations, because not affected by extraction and purification procedures used in the present study, also are assumed to have had lengths that more closely represented those of unaltered particles.

The three serological assays (microprecipitin, DAS-ELISA, and immunodotblot) showed a similar serological relationship of MDMV-O to the other MDMV strains. The DAS-ELISA used did not involve enzyme-conjugated viral antibodies and thus most likely had a broader reactivity than if such conjugated antibodies had been used. In these assays, MDMV-O was only distantly

TABLE 3. Serological reactivity of purified maize dwarf mosaic virus (MDMV) strains A, B, D, F, and O with their respective antibodies as determined by the immunodotblot assay[a]

Antigen[b]	Experiment no./reactivity (SDI_3)[c]	Antibodies to: MDMV-A	MDMV-B	MDMV-D	MDMV-F	MDMV-O
MDMV-A	1	162[d]	0[e]	486	486	54
	2	162	6	486	486	18
	SDI_3	0	−4.67	1.0	1.0	−4.5
MDMV-B	1	0	486	18	6	0
	2	6	486	6	18	0
	SDI_3	−3.67	0	−4.5	−4.5	
MDMV-D	1	486	2	1,458	1,458	2
	2	162	2	1,458	1,458	18
	SDI_3	0.5	−5	0	0	−5.67
MDMV-F	1	486	2	13,122	1,458	0
	2	162	2	1,458	1,458	18
	SDI_3	0.5	−5	1.33	0	−5.75
MDMV-O	1	0	0	0	18	4,374
	2	0	0	0	18	4,374
	SDI_3				−4.0	0

[a] The assay was performed as follows: dilutions of purified MDMV strains were blotted onto nitrocellulose; unoccupied sites were blocked with nonfat dry milk; antigen was detected with purified IgG; bound antibodies were detected with protein A-alkaline phosphatase conjugate; and the treated nitrocellulose was incubated over an agarose gel containing the enzyme substrate 5-bromo-4-chloro-3-indoyl phosphate.

[b] Each purified MDMV strain (206 μg/ml) was diluted in a twofold dilution step followed by 10 threefold dilution steps with Tris-buffered saline-Tween as the diluent.

[c] SDI_3 (serological differentiation index) denotes the number of threefold dilutions separating the mean of the reciprocals of the greatest dilutions giving a positive reaction for antibodies to a particular MDMV strain with a different MDMV strain from the mean of the reciprocals of the greatest dilutions giving a positive reaction for the same antibodies with the MDMV strain to which they were prepared.

[d] Reciprocal of the greatest dilution giving a blue dot on both the nitrocellulose membrane and substrate-containing agarose gel.

[e] 0 denotes no reaction.

related to MDMV strains A, B, D, E, and F. Its relationship to SCMV-A was also distant, but it was unrelated to SCMV-H. These MDMV strains previously had been reported to be serologically related to each other (12,29), and the two SCMV strains had been reported to be serologically related to other MDMV isolates (10,21,34,38,41). Our findings and those just cited suggest the following serogroups for U.S. strains of MDMV and SCMV: a) MDMV-A, -C, -D, -E, and -F; b) MDMV-B and SCMV-A, -B, -D, and -E; c) MDMV-O; d) SCMV-H, -I, and -M; and e) MDMV-KS1. However, the serological relationship of MDMV-O to MDMV-KS1 has not been ascertained, making their separation problematical.

The $s_{20,w}$ (168 S) and buoyant density (1.297 g/ml) of MDMV-O virions were within the range of values for these two characteristics reported for other MDMV isolates and strains, that is, 148–170 S and 1.254–1.423 g/ml, respectively (8).

Based on our finding that MDMV-O nucleic acid is degraded by RNase A and not by DNase I, we conclude that MDMV-O contains RNA. The RNA constitutes 6–7% of the particle weight. These characteristics are similar to those for MDMV-B (18) and are characteristic of potyviruses (19).

In our study, the dissociated capsid-protein subunits of MDMV-O migrated as two distinct bands often accompanied by a less distinct third band with M_r for the three subunits of 35.1, 33.0, and 30.5 kDa, respectively. Previously, Langham and Toler (27) reported a single M_r (37.9 kDa) for the capsid protein of their isolate of MDMV-O but did not show a photograph of a stained SDS-PAGE gel containing the MDMV-O capsid protein to confirm the presence of only a single subunit. Previously, Hill et al (18) reported that noncarboxymethylated MDMV-B capsid protein migrated as multiple bands in both anionic and cationic electrophoresis systems, but only as a single band when reduced and carboxymethylated. Reduction and carboxymethylation of our MDMV-O capsid protein did not eliminate the additional two bands. Nonreduced and noncarboxymethylated MDMV-B capsid protein in our study also migrated as three distinct bands. We did not test reduced, carboxymethylated MDMV-B capsid protein by SDS-PAGE. Although the capsid-protein subunit migration pattern for MDMV-O in SDS-PAGE more closely resembled that for MDMV-B, inclusion body morphology and serological relationships indicated greater similarity between MDMV-O and MDMV strains A and F, respectively, than MDMV-B.

For the capsid proteins of MDMV strains A, D, E, and F, we obtained a single band in agreement with several other earlier reports for these MDMV strains and several SCMV strains (13,22,27). Although MDMV-A gave a single capsid-protein subunit, the molecular weight of the subunit (M_r = 27.7 kDa) was less than that of the smallest of the three subunits (M_r = 30.0 kDa) of strains B and O; both of these subunits were possibly derived from the largest (M_r = approximately 35.0 kDa) of the subunits. Thus, it is speculated that the capsid protein of MDMV-A in our study was completely converted to the fastest (B) subunit.

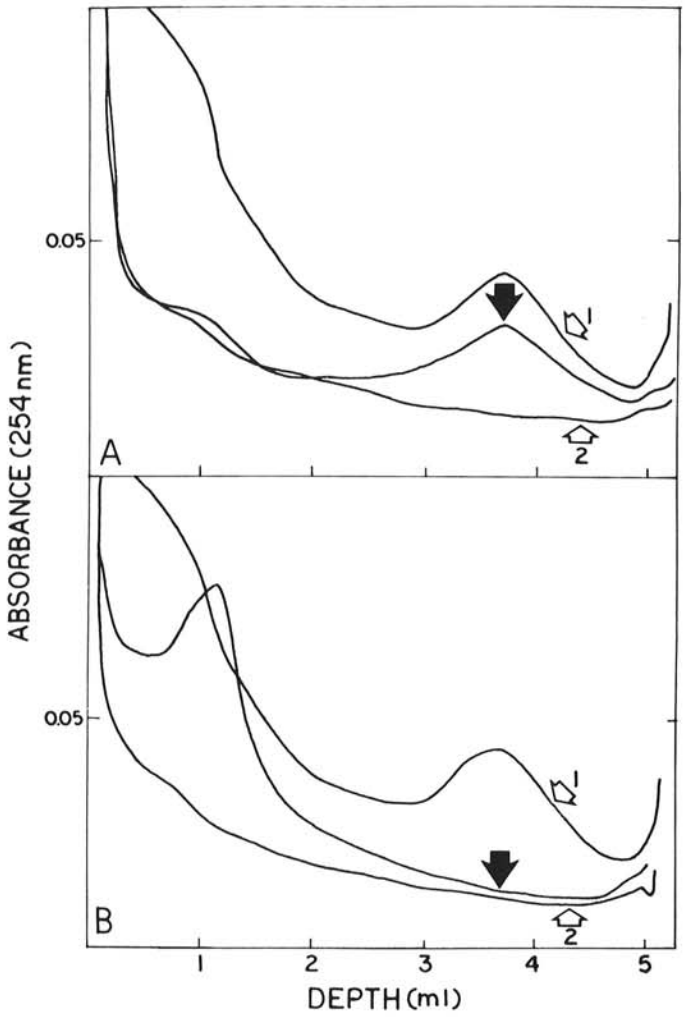

Fig. 2. Ultraviolet (254 nm) absorbance profile of centrifuged sucrose density gradients containing nucleic acid from virions of maize dwarf mosaic virus strain O treated with either DNase I or RNase A. **A,** Solid arrow points to the profile of DNase-treated nucleic acid; arrow 1 points to the profile of untreated nucleic acid; and arrow 2 points to the profile of a centrifuged gradient containing only the nuclease. **B,** Solid arrow points to the profile of RNase-treated nucleic acid; arrow 1 points to the profile of untreated nucleic acid; and arrow 2 points to the profile of a centrifuged gradient containing only the nuclease.

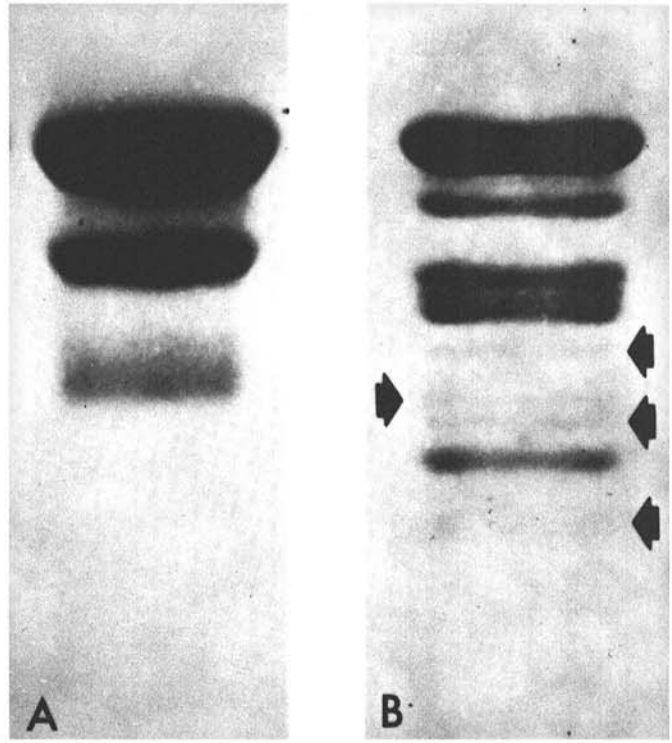

Fig. 3. Coomassie Blue R-250 stained capsid-protein subunits from maize dwarf mosaic virus strain O (MDMV-O) virions, CsCl purified from maize and dissociated in sodium dodecyl sulfate (SDS). Electrophoresis was (top to bottom) in an SDS-10% polyacrylamide gel. Bands are enlarged for better visualization. See Figure 4, lane 2 for relationship of MDMV-O capsid-protein bands to molecular weight markers. **A,** The capsid protein was electrophoresed within hours after dissociation of the virions in SDS, and the banding pattern is typical for preparations analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) for 11, 22, and 33 days of storage. **B,** The dissociated protein had been stored at 3 C in a sodium azide-containing buffer for 44 days before SDS-PAGE. Arrows indicate less distinct bands. The subunit bands in B showed greater separation than those in A, presumably because the capsid proteins in the two runs were electrophoresed at different times.

Although we did not estimate the M_r for strains D, E, and F, based on their positions relative to the bands for strains A, B, and O, they appeared to have capsid-protein molecular weights of about 33.0–35.0 kDa or equivalent to that of the M or T band of MDMV-O and MDMV-B.

Capsid-protein subunits of several other potyviruses have been reported to migrate as two (15,17,20,31,33) or three (16,32) bands in SDS-PAGE with M_r between 32.0 and 38.0 kDa for the largest protein and 26.0 and 30.0 kDa for the one or two smaller capsid proteins. Most authors (16,17,20,32,33) attribute the origin of the smaller protein(s) to limited proteolysis of the larger capsid-protein subunit by proteolytic enzymes of infected-plant or microbial origin. Hiebert and McDonald (16) suggested that the capsid protein of turnip mosaic virus converted from the slow to fast migrating form either in situ or during the initial stage in purification. If MDMV-O capsid protein was similarly converted by proteolysis to produce the two additional, faster migrating subunits, then this too must have occurred in situ or during the initial stage of purification. Further conversion of the slowest migrating subunit (T) to the other two subunits (M and B) was not observed for up to 34 days of capsid-protein storage under conditions that presumably removed proteolytic activity (purification of virions through CsCl) or inhibited its activity or development (addition of sodium azide and storage of the preparation at 3 C). However, several slightly faster migrating bands appeared to be associated with each of the three original bands for preparations stored for 44 or more days, giving evidence of partial degradation of each of these subunits after longer storage. Perhaps, this change was the result of the deterioration of sodium azide allowing microbial growth and development of proteolytic enzyme activity. If the two faster migrating bands (M and B) were the products of proteolysis of the slower migrating subunit, then specific cleavage sites on the larger protein to produce the former two subunits appear involved because the apparent molecular weights of the two faster migrating subunits were generally consistent for all the MDMV-O capsid-protein preparations. Also, because two faster migrating subunits were obtained, it is suggested that proteolysis occurs in two steps, the first producing the 33.0 kDa subunit and the second the 30.0 kDa subunit. Hiebert et al (17) reported the loss of two peptides after limited proteolysis of tobacco etch virus capsid protein, with a corresponding decrease in molecular weight from 32.0 to 26.0 kDa.

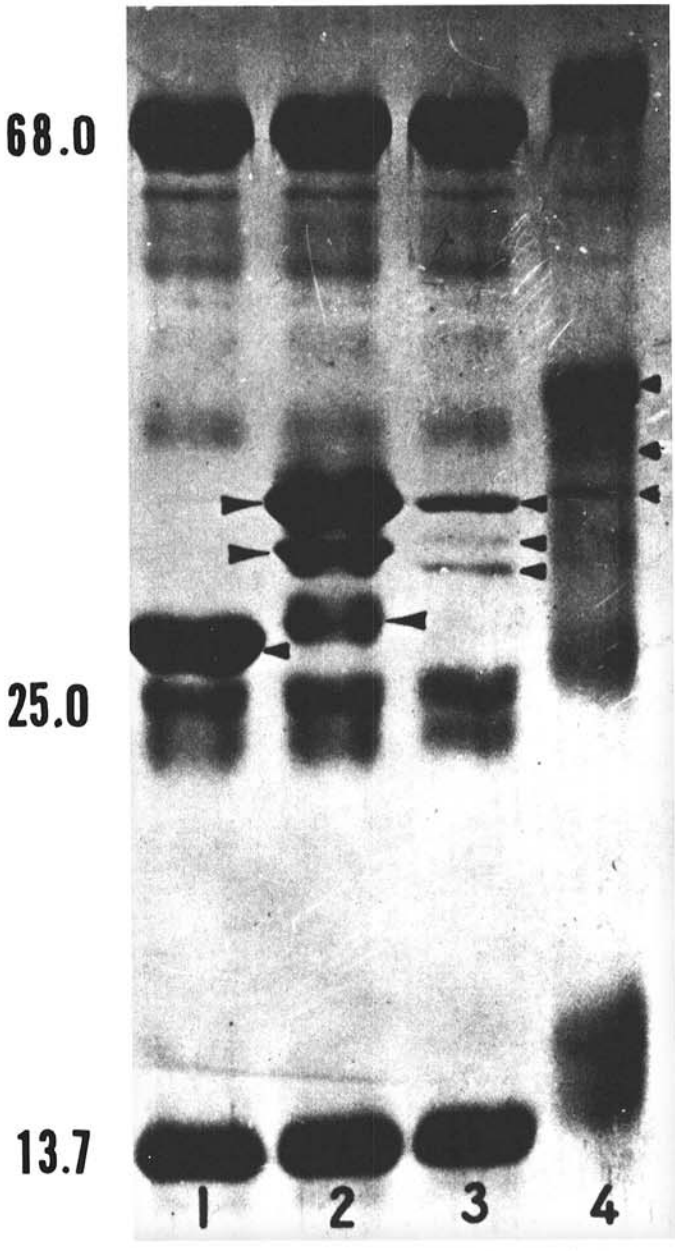

Fig. 4. Coomassie Blue R-250 stained capsid-protein subunits (arrows) from maize dwarf mosaic virus (MDMV) virions dissociated in sodium dodecyl sulfate (SDS) and electrophoresed in an SDS-10% polyacrylamide gel. (Left to right) noncarboxymethylated capsid-protein subunits from CsCl-purified MDMV strains A (lane 1), O (lane 2), and B (lane 3) and reduced, carboxymethylated MDMV-O capsid-protein subunits (lane 4). Molecular weight marker proteins, included in each lane, were (top to bottom) bovine serum albumin (molecular weight = 68 kilodaltons [kDa]), chymotrypsinogen (molecular weight = 25 kDa), and RNase A (molecular weight = 13.7 kDa). Multiple, unmarked protein bands, observed primarily in the upper one-third of the gel, represent multimers of both the molecular weight marker proteins and virion capsid-protein subunits.

Deletion of a portion of the capsid protein through enzyme-mediated proteolysis has been reported to alter serological reactivity of the protein for other potyviruses (16). However, in our study only some of the MDMV-O capsid protein appears to have degraded and our serological comparisons should reflect serological reactions for partially degraded and undegraded capsid protein of MDMV-O. However, the considerably smaller capsid protein identified for MDMV-A (27.7 kDa) in our study indicates that all of the capsid protein of this strain was degraded to the fastest migrating form, thereby possibly deleting additional serologically reactive sites. The degree of presumed degradation of the capsid proteins of the virions involved in our assays was not evaluated, and thus further serological relatedness may have gone undetected.

Other explanations to account for the three capsid-protein subunits of MDMV-O are possible. For example, these three capsid-protein subunits may have resulted from termination of translation in the synthesis of the capsid protein at three distinct

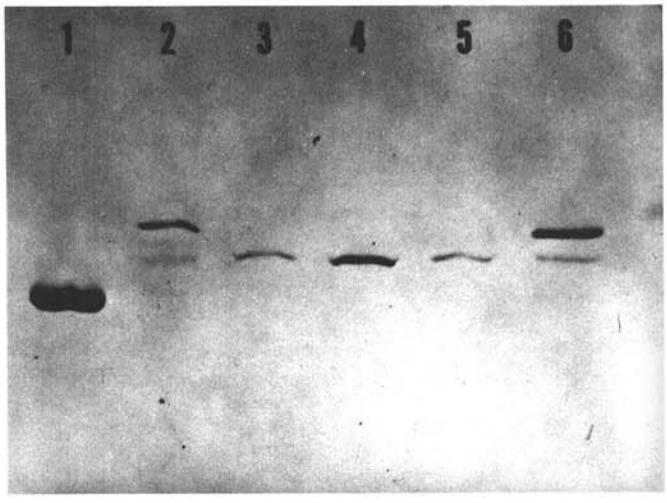

Fig. 5. Coomassie Blue R-250 stained capsid-protein subunits from maize dwarf mosaic virus (MDMV) virions, CsCl-purified from maize and dissociated in sodium dodecyl sulfate (SDS). Electrophoresis was (top to bottom) in an SDS-10% polyacrylamide gel. MDMV strains are A (lane 1), B (lane 2), D (lane 3), E (lane 4), F (lane 5), and O (lane 6).

sites on the mRNA. Another explanation for their origin would be that our MDMV-O is a mixture of three MDMV-O isolates, each with a capsid protein of a different M_r. Although evidence for such differences among isolates of MDMV and SCMV has been presented recently (22), the capsid proteins among these isolates varied between 34.4 to 39.7 kDa, values considerably larger than what we observed for the fastest of the three MDMV-O capsid-protein subunits. Such a mixture also might explain the differences in the relative amount of the three capsid bands. The source or origin of the three capsid-protein subunit bands observed for MDMV-B might be similarly explained.

As a further alternate explanation, the multiple bands may have been the result of a mixture of a single capsid-protein subunit with host plant proteins contaminating the virion preparation. However, the consistent appearance of at least the two (T and M) slower migrating bands in the SDS-PAGE gels and their presence in the preparations from the three infected hosts (maize, oats, and sorghum) suggest that they are not host derived but rather are of viral origin. Finally, these proteins may be charge isomers resulting from differential SDS binding to the capsid-protein subunits due to minor changes in their amino acid composition (14), as suggested for several other potyviruses (15,32).

Although further study of the MDMV-O capsid protein is required before any of the above possibilities as to the origin of the multiple capsid-protein subunits can be definitely eliminated, the most probable of the interpretations seems to be enzyme-mediated proteolysis in vivo or in the initial stage of virion purification.

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