

Serological Differentiation of Maize Dwarf Mosaic Potyvirus Strains A, D, E, and F by Electro-Blot Immunoassay

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ABSTRACT

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Antiserum recovered 1 wk after a single immunization (As-1wk) with maize dwarf mosaic potyvirus (MDMV) strain A, D, E, or F reacted strongly with the respective homologous capsid protein but weakly or not at all with the corresponding homologous core-capsid protein in electro-blot immunoassays (EBIA). The antisera also reacted with the heterologous capsid proteins of these strains but not with capsid proteins from other members of the potyvirus group (i. e., sugarcane mosaic virus strain MDB [SCMV-MDB], formerly MDMV-B, and johnsongrass mosaic virus strain O [JGMV-O], formerly MDMV-O). As-1wk to the latter two viruses reacted only with the respective homologous capsid protein. Antiserum recovered four or more weeks after one to six immuniza-

tions (As-swk) with MDMV-A, -D, -E, -F, SCMV-MDB, or JGMV-O reacted with the capsid proteins and core-capsid proteins of all the viruses and strains. Cross-absorbing As-swk to strain A, D, E, or F with heterologous capsid protein (except strain D) only eliminated reactions with the cross-absorbing strain. Cross-absorbing As-swk to strains A, E, or F with MDMV-D capsid protein eliminated reactions with the capsid protein of one or more strains in addition to strain D. We conclude that strains A, D, E, and F contain unique epitopes located on the portion of the capsid protein removed by endopeptidase. This is the first demonstration of serological differences among MDMV strains A, D, E, and F, which previously were differentiated only biologically.

Strains A, D, E, and F of the maize dwarf mosaic virus (MDMV) from the United States have been differentiated principally by symptoms incited on maize (*Zea mays* L.) and by other biological properties (13). However, environmental conditions can make recognition of the distinguishing symptoms of these strains problematic (8). Thus, discovery of a characteristic that more accurately and consistently differentiates them would be useful.

The serology of the capsid proteins of MDMV and sugarcane mosaic virus (SCMV) have been studied extensively to identify viruses and differentiate strains (7,8,11,15,17,19). However, antisera, mostly obtained after lengthy immunizations, when tested by enzyme linked immunosorbent assay (ELISA) (7,15) have accurately separated only some of the MDMV/SCMV strains. In electro-blot immunoassay (EBIA), the antisera have even broader cross-reactivity with these strains and other potyviruses, making separation problematic (17,19). In contrast, antisera recovered after short periods of immunization have shown more specificity toward individual potyviruses and their strains (19). Also, high-titered antisera, obtained after lengthy immunizations, to MDMV and other viruses have shown greater specificity toward strains when cross-absorbed with virions of heterologous strains than antisera that were not cross-absorbed (4,21,25). To date, MDMV-A, -D, -E, and -F have been separated serologically by several methods from isolates formerly designated as MDMV-B and -O but not from each other (11,15,19). The serological differences of MDMV-B and -O as well as significant differences in sequence homology led to their reclassification as strains of SCMV (SCMV-MDB) and the newly named johnsongrass mosaic virus (JGMV-O), respectively (19,23). In this report, we present evidence that antisera raised to intact virions and recovered either shortly after a single immunization or at later times and cross-absorbed with heterologous capsid proteins contain strain-specific anti-

bodies when tested by EBIA, demonstrating that MDMV strains A, D, E, and F are serologically distinct. These findings were reported previously (12).

MATERIALS AND METHODS

Virus and virus strains. MDMV-A, -D, -E, -F, SCMV-MDB, and JGMV-O were maintained in sorghum (*Sorghum bicolor* (L.) Moench) cv. Sart in separate greenhouses at 21–30 C. MDMV-D, -E, and -F were obtained from R. Louie (USDA-ARS, Wooster, OH). MDMV-C had been lost and was not tested. The identity of all MDMV strains was confirmed by host range and differential symptoms (3,13,14). MDMV-A, -D, -E, and -F infected johnsongrass (*Sorghum halepense* (L.) Pers.); each incited characteristic symptoms on maize-inbred N20 and Sart sorghum as described on N20 maize by Louie and Knoke (13). SCMV-MDB did not infect johnsongrass. JGMV-O infected both johnsongrass and Garland oats (*Avena sativa* L.).

Virus purification. Virions were purified using the Tris-citrate-buffer method (15). Virus purity and yield were estimated spectrophotometrically (15). Mean absorbance ratios of $A_{260\text{nm}}/A_{280\text{nm}}$ for partially purified virions varied from 1.30 to 1.35 and were within the previously published range for highly purified MDMV (2,6,10,15,20). Virus yields gave values (12–30 μg per gram of infected tissue) similar to those reported previously (15,20).

Antisera source. Antisera for all viruses and strains except MDMV-A were raised by immunizing New Zealand white rabbits with purified, intact virions as follows. Each rabbit was immunized by a single intramuscular injection of 100–150 μg of purified virions suspended in 500 μl of Hepes-buffered saline (20 mM Hepes in 0.15 M NaCl, pH 7.3) and emulsified with an equal volume of Freund's incomplete adjuvant (Gibco Laboratories, Life Technologies, Inc., Chagrin Falls, OH). Antisera were recovered from blood collected from each rabbit 1, 2, 3, and 4 wk after immunization, were diluted with an equal volume of glycerol, and were stored at –20 C. The MDMV-A antiserum was described previously and was collected 1, 2, 3, and 4 wk

after a single immunization and several weeks after repeated immunizations (15). Late-bleeding antisera for the other strains were produced similarly to that produced for MDMV-A (D. T. Gordon, unpublished data).

Capsid- and core-capsid-protein preparation. Core-capsid proteins were prepared by incubating 1–2 mg of purified virions suspended in 0.01 M Tris-citrate buffer (pH 7.0) with 6 μ g of lysyl endopeptidase (Wako Chemical, Dallas, TX) per milligram of virus for 30 min at room temperature (17). The mixture was then centrifuged in a Beckman SW60Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) at 407,000 *g* for 19 min at 5 C. The supernatant was discarded and the pelleted virions were resuspended in the original volume of 0.01 M Tris-citrate buffer (pH 7.0) plus 0.02% sodium azide.

Capsid-protein molecular-mass determinations. The molecular masses of capsid and core-capsid proteins were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using the Laemmli system (9), in a 12.5% gel with a Mini-Protean II dual slab cell (Bio-Rad Laboratories, Richmond, CA). Molecular masses of capsid and core-capsid proteins were estimated using a regression equation (24).

Electro-blot immunoassay (EBIA). Capsid and core-capsid proteins (0.5 μ g per lane) were separated by SDS-PAGE and transferred to PVDF (polyvinylidene-difluoride) membranes (Millipore Corporation, Bedford, MA) using a mini trans-blot electrophoretic transfer cell (Bio-Rad Laboratories). After blocking with Tris-buffered saline (TBS) containing 5% nonfat dry milk (NFDM) and 0.02% Na_3N for 1 h or overnight at room temperature, the membranes were probed for 2 h or overnight on a shaker at room temperature with specific antisera diluted in TBS containing 0.05% Tween 20 (TBS-T), 1% NFDM, and 0.02% Na_3N . The membranes were then incubated with alkaline phosphatase-conjugated goat-antirabbit antibodies and subsequently treated with a modified substrate solution (16). Membranes were washed with TBS containing 0.1% NFDM and 0.02% Na_3N three times between each of the above treatments.

Tests of antisera specificity. To test for terminus-specific antibodies (i.e., antibodies that reacted with the homologous capsid

protein but not with the homologous core-capsid protein) using EBIA, membranes containing 0.5 μ g of capsid protein or core-capsid protein per lane were incubated with the homologous antiserum collected 1, 2, and 4 wk after one immunization and were diluted 1:10,000.

To determine the virus and strain specificity of early-bleeding antisera (As-1wk) and late-bleeding antisera (As-swk), membranes containing the capsid proteins of the four MDMV strains, the

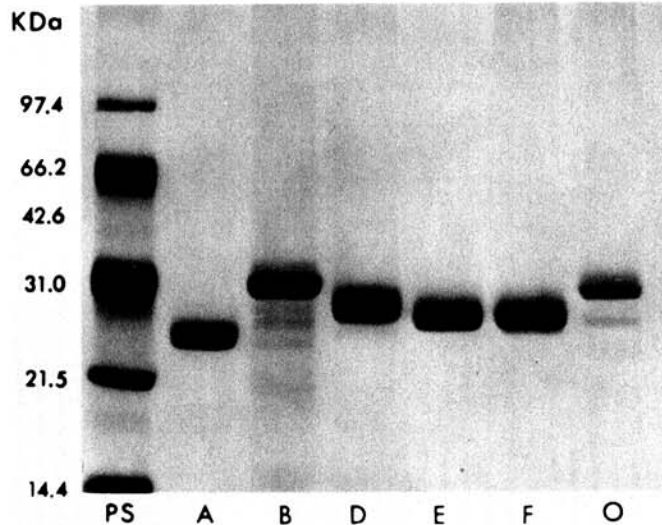


Fig. 1. Banding pattern of silver-stained capsid proteins of purified maize dwarf mosaic virus (MDMV) strains A, D, E, F, sugarcane mosaic virus (SCMV) strain MDB, and johnsongrass mosaic virus (JGMV) strain O following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% polyacrylamide gels. Left lane contained: 0.5 μ g of each of the silver-stained low molecular-mass standard proteins (PS): phosphorylase b (97.4 kDa); bovine serum albumin (66.2 kDa); ovalbumin (42.6 kDa); bovine carbonic anhydrase (31.0 kDa); soybean trypsin inhibitor (21.5 kDa); and lysozyme (14.4 kDa). Calculated molecular-mass values of bands or main bands are shown in parentheses. Remaining lanes from left to right contained the capsid proteins of MDMV strain A, SCMV strain MDB, MDMV strains D, E, F, and JGMV strain O, respectively.

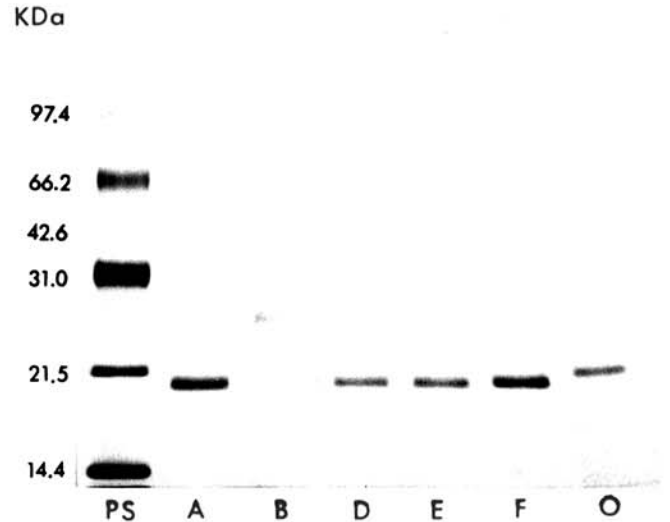


Fig. 2. Banding patterns on 12.5% polyacrylamide gel of silver-stained core-capsid proteins of maize dwarf mosaic virus (MDMV) strains A, D, E, F, sugarcane mosaic virus (SCMV) strain MDB, and johnsongrass mosaic virus (JGMV) strain O obtained by mild lysyl-endopeptidase treatment of intact virions following SDS-PAGE. Left lane contained 0.5 μ g of the silver-stained low molecular-mass standard proteins (PS): phosphorylase b (97.4 kDa); bovine serum albumin (66.2 kDa); ovalbumin (42.6 kDa); bovine carbonic anhydrase (31.0 kDa); soybean trypsin inhibitor (21.5 kDa); and lysozyme (14.4 kDa). Calculated molecular-mass values of bands or main bands are shown in parentheses. Remaining lanes from left to right contained the core-capsid proteins of MDMV strain A, SCMV strain MDB, MDMV strains D, E, F, and JGMV strain O, respectively.

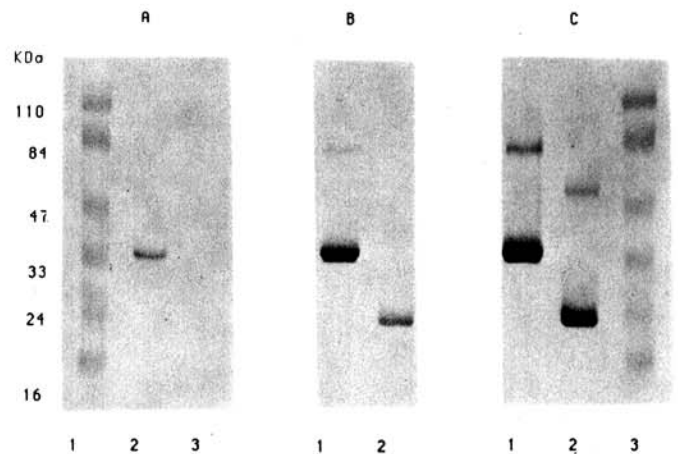


Fig. 3. Reaction of maize dwarf mosaic virus strain F (MDMV-F) capsid protein and core-capsid protein with homologous antisera from bleedings A, 1 wk, B, 2 wk, and C, 4 wk after primary immunization in electro-blot immunoassay. A, Lane 1 contained 0.62 μ g of prestained molecular-mass standard proteins (PS): phosphorylase b (110 kDa); bovine serum albumin (84 kDa); ovalbumin (47 kDa); bovine carbonic anhydrase (33 kDa); soybean trypsin inhibitor (24 kDa); and lysozyme (16 kDa). Calculated molecular-mass values are shown in parentheses. Lanes 2 and 3 contained 0.5 μ g of capsid protein and core-capsid protein, respectively. B and C, Lanes 1 and 2 contained capsid protein and core-capsid protein, respectively.

SCMV-MDB strain, and the JGMV-O strain were probed with each antiserum separately. To test the specificity of cross-absorbed MDMV-A, -D, -E, and -F antisera, 0.5 μ l of aliquots of each antiserum were incubated individually with 500 μ g of each of the heterologous capsid proteins and then tested by EBIA for reactivity with the four capsid proteins. Control antisera, aliquots incubated without capsid proteins, were included in these tests.

RESULTS

Molecular masses of capsid proteins. The capsid proteins of MDMV-A, -D, -E, and -F in SDS-PAGE migrated as single bands with the following molecular masses: MDMV-A, 27.7 kDa; -D, 31.1 kDa; -E, 30.5 kDa; and -F, 30.5 kDa. In contrast, the capsid proteins of SCMV-MDB and JGMV-O each migrated as a main band, with molecular masses of 36.2 and 36.9 kDa, respectively (Fig. 1), plus one or two prominent faster-migrating bands. The core-capsid proteins of all MDMV strains as well as JGMV-O migrated as single bands in SDS-PAGE (Fig. 2), with molecular masses of 21.5 kDa; SCMV-MDB migrated as a single band but had a molecular mass of 29.5 kDa.

EBIA identification of antibodies, from the first, second, and fourth bleedings, reactive with the termini and cores of capsid proteins. In EBIA, the capsid protein but not the core-capsid protein of MDMV-F reacted with the homologous antiserum from the early bleeding (As-1wk), whereas both capsid protein and core-capsid protein reacted with antisera collected from later (2- and 4-wk) bleedings (Fig. 3). The core-capsid protein reaction with antiserum from the 2-wk bleeding was weaker than was the reaction from the 4-wk bleeding. Results were similar for antisera to the other MDMV strains, SCMV-MDB, and JGMV-O (data not shown).

Identification of terminus-specific antibodies from early-bleeding antisera. As further evidence that terminus-specific antibodies were present in the early-collected antisera, each early-bleeding antiserum (As-1wk) in EBIA reacted strongly with the homologous capsid proteins at antisera dilutions up to 1:19,200 but failed to react or reacted only weakly, at antiserum dilutions of 1:75–1:300, with the homologous core-capsid proteins (Table 1).

In contrast, 4-wk antisera reacted strongly with both homologous capsid and core-capsid proteins at a dilution of 1:10,000, the only dilution tested.

Strain specificity of antisera from early and late bleedings. Early-bleeding antiserum to MDMV-A (Fig. 4), -D, -E, and -F (data not shown) reacted with the capsid proteins of MDMV strains A, D, E, and F but not with those of SCMV-MDB and JGMV-O. Early-bleeding antisera to SCMV-MDB and JGMV-O reacted only with their homologous capsid proteins (Fig. 4). In contrast, late-bleeding antiserum to MDMV-A reacted with the capsid proteins of all MDMV, SCMV-MDB, and JGMV-O (Fig. 5). The late-bleeding antisera to MDMV-D, -E, -F, SCMV-MDB, and JGMV-O also reacted with the capsid proteins of all MDMV strains and viruses (data not shown).

Strain specificity of cross-absorbed, late-bleeding antisera to MDMV-A, -D, -E, and -F. MDMV-A, -D, -E, or -F late-bleeding antiserum cross-absorbed with heterologous capsid protein of strains A, E, or F did not react with the capsid protein used to cross-absorb but did react with the other heterologous and homologous capsid proteins (Table 2). In contrast, late-bleeding antiserum to MDMV-A, -E, or -F cross-absorbed with MDMV-D capsid protein showed a different pattern of reactivity. MDMV-A antiserum cross-absorbed with MDMV-D capsid protein reacted strongly with MDMV-A capsid protein, weakly with MDMV-E capsid protein, and not at all with MDMV-D and MDMV-F capsid proteins. MDMV-E antiserum cross-absorbed with MDMV-D capsid protein reacted with the capsid proteins of MDMV-E and MDMV-F but not with those of MDMV-A and MDMV-D. Finally, MDMV-F antiserum cross-absorbed with MDMV-D capsid protein reacted only with MDMV-F capsid protein. Antisera that were not cross-absorbed reacted with their respective homologous capsid proteins and all three heterologous capsid proteins.

DISCUSSION

The capsid proteins of MDMV-A, -D, -E, -F, SCMV-MDB, and JGMV-O migrated in SDS-PAGE as previously reported (8,11,15,22). The variation in the molecular masses of these capsid

TABLE 1. Reactivity of dilutions of early-bleeding^a antisera to maize dwarf mosaic virus (MDMV) strains A, D, E, F, sugarcane mosaic virus (SCMV) strain MDB, and johnsongrass mosaic virus (JGMV) strain O with the homologous capsid protein (CP) and core-capsid protein (CCP)^b in electro-blot immunoassay^c

Strain capsid protein	Antiserum dilutions									Control ^d
	1:75	1:150	1:300	1:600	1:1,200	1:2,400	1:4,800	1:9,600	1:19,200	
MDMV-A										
CP	+	+	+	+	+	+	+	+	+	+
CCP	±	–	–	–	–	–	–	–	–	–
SCMV-MDB										
CP	+	+	+	+	+	+	±	±	±	+
CCP	±	±	±	–	–	–	–	–	–	–
MDMV-D										
CP	+	+	+	+	+	+	+	+	+	+
CCP	±	±	±	–	–	–	–	–	–	–
MDMV-E										
CP	+	+	+	+	+	+	+	+	+	+
CCP	±	–	–	–	–	–	–	–	–	–
MDMV-F										
CP	+	+	+	+	+	+	+	+	+	+
CCP	–	–	–	–	–	–	–	–	–	–
JGMV-O										
CP	+	+	+	+	+	+	+	+	+	+
CCP	±	±	–	–	–	–	–	–	–	–

^a 1 wk after a single injection of purified virions.

^b CCPs were obtained from intact virions treated with lysyl endopeptidase (6 μ g enzyme/1–2 mg of purified virus).

^c 0.5 μ g CPs and CCPs from MDMV-A, -D, -E, -F, SCMV-MDB, and JGMV-O were electrophoresed by SDS-PAGE and transferred to PVDF membranes, which were cut into strips containing both CP and CCP and probed with the homologous antisera from the early bleeding at different dilutions.

^d Homologous antisera from the fourth (4-wk) bleeding, diluted 1:10,000.

+ = positive serological reaction (i.e., CP or CCP was immunostained).

– = negative serological reaction (i.e., CP or CCP was not immunostained).

± = weak serological reaction (i.e., CP or CCP was weakly immunostained).

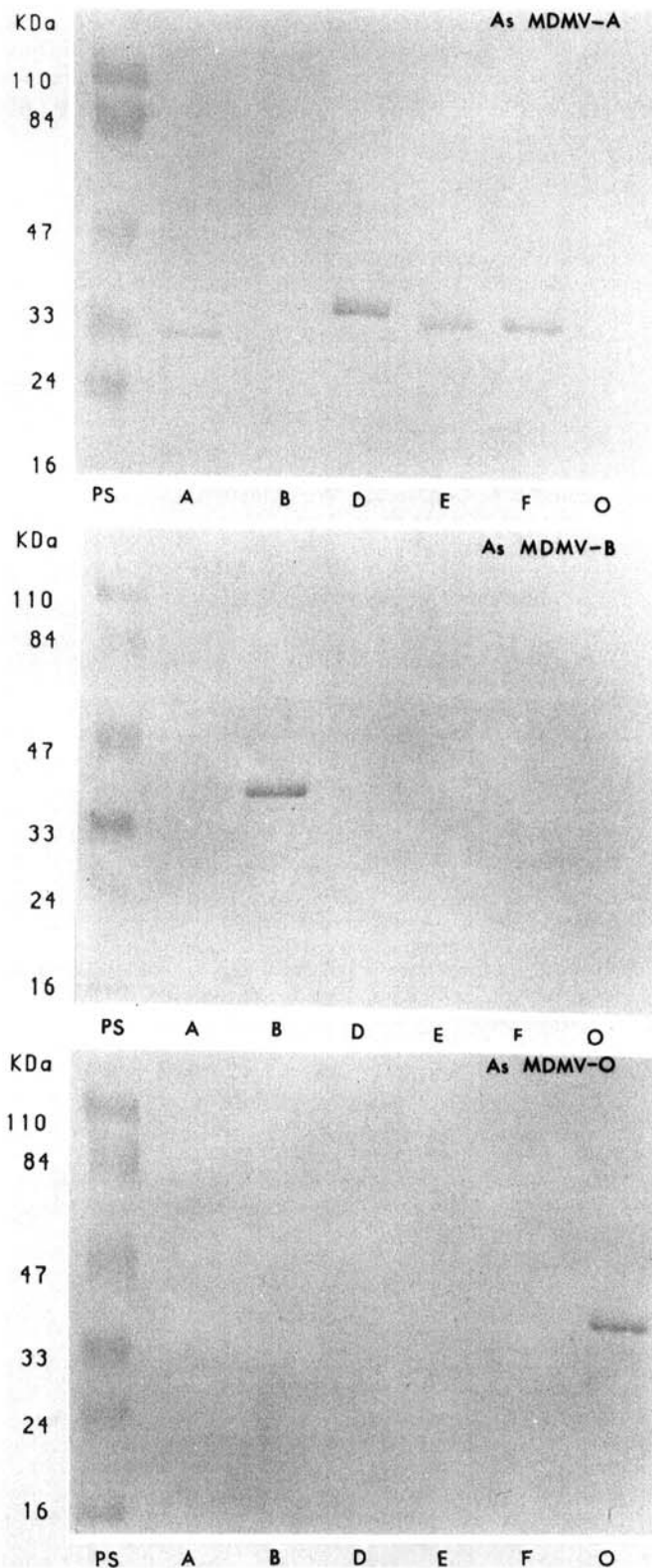


Fig. 4. Reactions of early-bleeding (1 wk after a single injection of purified virions) antisera to maize dwarf mosaic virus (MDMV) strain A (labeled MDMV-A), sugarcane mosaic virus (SCMV) strain MDB (labeled MDMV-B), and johnsongrass mosaic virus (JGMV) strain O (labeled MDMV-O) with homologous and heterologous capsid proteins in electro-blot immunoassay. Membranes were probed with MDMV-A, SCMV-MDB, and JGMV-O antisera from the early bleeding, respectively, diluted 1:5,000. The first lanes contained molecular-mass standard proteins (PS): phosphorylase b (110 kDa); bovine serum albumin (84 kDa); ovalbumin (47 kDa); bovine carbonic anhydrase (33 kDa); soybean trypsin inhibitor (24 kDa); and lysozyme (16 kDa). Calculated molecular-mass values are shown in parentheses. Lanes 2-7 contained 0.5 μ g per well of MDMV-A, SCMV-MDB, MDMV-D, -E, -F, and JGMV-O capsid proteins, respectively.

proteins was attributed to variable sizes of N-termini, as demonstrated for other potyviruses (1,5,18). Speculations on explanations for the multiple bands of SCMV-MDB and JGMV-O capsid proteins have been presented previously (15). Proteolytic treatment of these capsid proteins provided core capsid proteins that migrated as single bands with the same molecular masses (21.5 kDa), except for SCMV-MDB, which had a higher core capsid-protein molecular mass (29.5 kDa), perhaps indicating incomplete removal of the surface-exposed, terminal amino-acid sequences

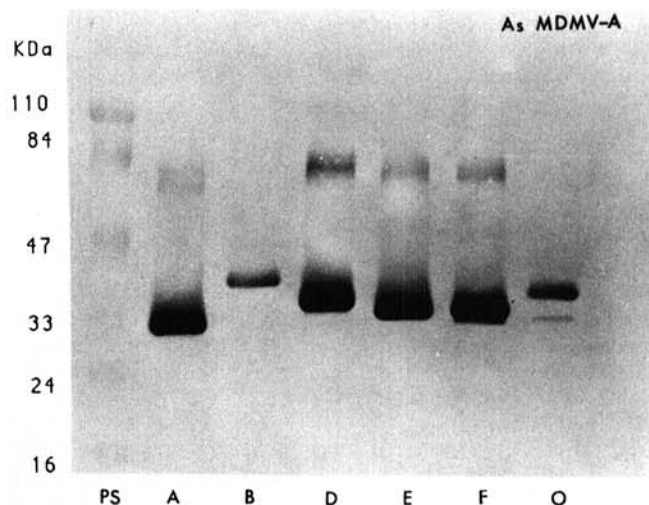


Fig. 5. Reaction of late-bleeding antiserum (dilution 1:5,000) to maize dwarf mosaic virus (MDMV) strain A with homologous and heterologous capsid proteins in electro-blot immunoassay. Lane 1 contained molecular-mass standard proteins (PS): phosphorylase b (110 kDa); bovine serum albumin (84 kDa); ovalbumin (47 kDa); bovine carbonic anhydrase (33 kDa); soybean trypsin inhibitor (24 kDa); and lysozyme (16 kDa). Calculated molecular-mass values are shown in parentheses. Lanes 2-7 contained 0.5 μ g per well of MDMV-A, SCMV-MDB, MDMV-D, -E, -F, and JGMV-O capsid proteins, respectively.

TABLE 2. Reactions of maize dwarf mosaic virus (MDMV) strains A, D, E, and F, late-bleeding antisera, cross-absorbed with heterologous capsid proteins (CPs), with homologous and heterologous CPs^a in electro-blot immunoassay

Antiserum	Cross-absorbing MDMV strain CP	Reaction with CP from MDMV ^b strain			
		A	D	E	F
MDMV-A	D	+	-	±	-
	E	+	±	-	±
	F	+	+	+	-
	none	+	+	+	+
MDMV-D	A	-	+	+	+
	E	+	+	-	+
	F	+	+	+	-
MDMV-E	A	-	+	+	+
	D	-	-	+	+
	F	+	+	+	-
	none	+	+	+	+
MDMV-F	A	-	+	+	+
	D	-	-	-	+
	E	+	+	-	+
	none	+	+	+	+

^a0.5 μ g of CP from MDMV-A, -D, -E, or -F was electrophoresed by SDS-PAGE, transferred to PVDF membranes, and probed with MDMV-A, -D, -E, or -F antisera cross-absorbed with a heterologous CP or with antisera not cross-absorbed (none).

^b+ = positive serological reaction (i.e., CP or CCP was immunostained). - = negative serological reaction (i.e., CP or CCP was not immunostained).

± = weak serological reaction (i.e., CP or CCP was weakly immunostained).

during proteolysis. Allison et al (1), Hiebert et al (5), and Shukla et al (17) reported that proteolytic treatment of potyviruses reduced their capsid protein to a trypsin-resistant core of 27, 26–29, and 30 kDa, respectively. The differences in core capsid-protein molecular masses among our isolates and those previously reported may have resulted from the use of different enzymes, percentages of polyacrylamide gels, molecular mass-marker proteins, and/or differences in core capsid-protein molecular masses among virus isolates.

Early-bleeding antisera (As-1wk) to all viruses reacted strongly with homologous capsid proteins but weakly or not at all with homologous core-capsid proteins, indicating a preponderance of terminus antibodies. In contrast, antisera from the 2- and 4-wk bleedings reacted strongly with both homologous capsid and core-capsid proteins, indicating a substantial increase in core-reacting antibodies. Shukla et al (19) reported that antibodies to MDMV-A from “early” collections (4 wk) reacted with the capsid protein of the homologous virus but weakly or not at all with the capsid proteins of other potyviruses, including some tested in our study. Further, their MDMV-A antiserum from 8-, 11-, and 13-wk bleedings reacted strongly, as expected, with the capsid proteins of all MDMV/SCMV strains tested by EBIA (19). Similarly, Tremaine and Wright (21) reported increased antisera cross-reactivity between two strains of southern bean mosaic virus with increased lengths of immunization.

Early-bleeding antisera (As-1wk) tested using EBIA separated our MDMV, SCMV-MDB, and JGMV-O strains into three distinct groups: 1) strains A, D, E, and F; 2) SCMV-MDB; and 3) JGMV-O. However, MDMV-A, -D, -E, and -F were not separated by these antisera. Similar results were reported by McDaniel and Gordon (15) for MDMV-A, -D, and -F (MDMV-E was not tested), using ELISA and dot-blot immunoassay (DBIA) with antisera to virions collected after several injections, and by Shukla et al (19), using EBIA with antisera to MDMV-A, SCMV (MDMV-B), and JGMV (MDMV-O) cross-absorbed with MDMV-A core-capsid protein. Our EBIA tests involving late-bleeding antisera did not allow serological separation of any of these strains and viruses, unlike the report by McDaniel and Gordon (15) who used only ELISA and DBIA. Our results indicated that these strains and viruses had some core capsid-protein epitopes in common, whereas some of the terminal epitopes were unique to the strains in the three groups. However, when late-bleeding antisera to MDMV-A, -D, -E, and -F were cross-absorbed with heterologous capsid proteins to remove cross-reactive core and N- and C-terminus-specific antibodies, serological separation of these strains was demonstrated. The cross-absorbed antisera of Shukla et al (19) failed to separate these four strains, presumably because the cross-absorbing antigen, MDMV-A core capsid protein, lacked the N-terminus to remove antibodies cross-reactive with this region of the capsid protein. In our study, antisera cross-absorbed with heterologous capsid proteins retained reactivity with capsid proteins of one or more of the remaining strains not used in the cross-absorption, indicating that some of the terminal epitopes were not shared by these strains (i.e., there was a serological difference among strains A, D, E, and F). Among strains MDMV-A, -D, -E, and -F, MDMV-D appeared to have the largest number of shared-terminal epitopes and showed the least amount of serological difference, because heterologous antisera cross-absorbed with MDMV-D capsid protein lost reactivity to one or more of the heterologous capsid proteins not used to cross-absorb the antisera. In contrast, when these antisera were cross-absorbed with the capsid protein of each of the remaining heterologous strains, only reactivity with the capsid protein used to cross-absorb was eliminated. Although we were able to demonstrate serological differences among MDMV strains A, D, E, and F by using cross-absorbed antisera, this approach does not allow for routine serological assay of infected samples to detect the individual strains.

The above results show serological differences between MDMV-A, -D, -E, and -F that correspond to the biological differences reported previously by Louie and Knoke (13). Our results also show that MDMV, SCMV-MDB, and JGMV-O are clearly re-

lated serologically as well as biologically and physically. Thus, we believe the recently adopted nomenclature (three distinct viruses) (19) does not reflect these relationships, whereas the former nomenclature (strains of one virus) more accurately reflects the serological and biological relationships exhibited by these isolates among themselves and with other potyviruses.

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